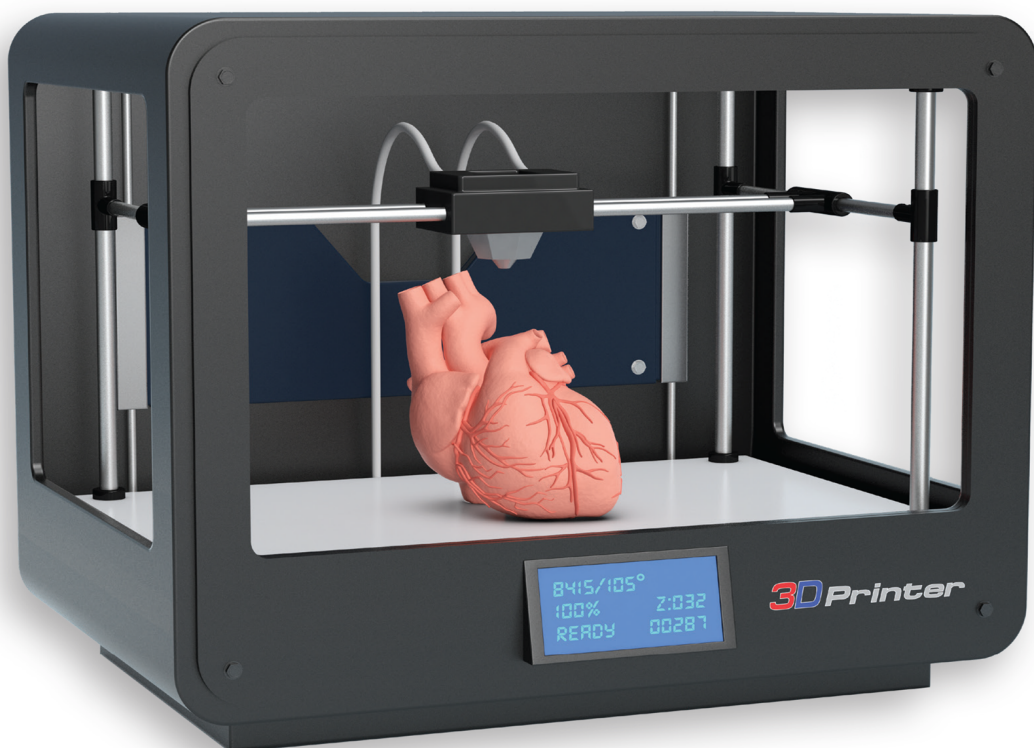


edited by
Ali Khademhosseini
Gulden Camci-Unal

CRC Press Series in
Regenerative Engineering

3D BIOPRINTING IN REGENERATIVE ENGINEERING

Principles and Applications



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3D Bioprinting in Regenerative Engineering

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Chapter 1 Principles and applications of bioprinting

A. Skardal

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1.1 Introduction

Bioprinting has emerged as a flexible tool in regenerative medicine with potential in a variety of applications. Bioprinting is a relatively new field within biotechnology that can be described as robotic additive biofabrication that has the potential to build or pattern viable organ-like or tissue structures in three dimensions.¹ In general, bioprinting uses a computer-controlled three-dimensional (3D) printing device to accurately deposit cells and biomaterials into precise geometries with the

goal being the creation of anatomically correct biological structures. Generally, bioprinting devices have the ability to print cell aggregates, cells encapsulated in hydrogels or viscous fluids, or cell-seeded microcarriers—all of which can be referred to as *bioink*—as well as cell-free polymers that provide mechanical structure or act as placeholders.^{2,3} Biologically inspired, physiologically relevant computer-assisted designs can be used to design and guide the placement of specific types of cells and materials into precise, planned geometries that mimic the architecture of actual tissue construction,⁴ which can subsequently be matured into functional tissue constructs or organs.^{5,6}

There is already a massive shortage of donor organs for implantation in patients.^{7,8} For example, as of July 2017, over 117,000 patients were still on waiting lists for donor organs, and there are only 8,096 currently identified available donors.⁹ Today, most transplants have high success rates. Kidney, pancreas, liver, intestine, and heart transplants have more than 80% 1-year survival rates and more than 70% 5-year survival rate (with the exception of intestine).¹⁰ These statistics illustrate the demand for an increased organ supply, as well as the fact that implanting organs is a useful and effective therapy. Furthermore, while preclinical testing of candidate drugs in animals is well established, it is neither particularly efficient nor is it always predictive of the clinical outcome in humans.¹¹ 3D human tissues, not animal tissues, are logically the most appropriate models for screening drugs meant for humans and investigating diseases afflicting humans. Bioprinted tissues and organs have the potential to address the need for implantable tissues for patients waiting on donor lists and testable human tissues for research and development.

1.2 Bioprinting—then and now

Bioprinting is a young scientific undertaking when compared with many other areas of research and technological development. Yet now, it has been around for nearly a decade and a half. In that time, the field has made extraordinary advances in some respects, but remains quite stagnant in others. As such, it makes sense for those of us who toil on this exciting field to compare where we are now to where we were when bioprinting was in infancy, thereby identifying the limitations and technical challenges that remain to be overcome before bioprinting can be fully scaled to biomanufacturing levels.

1.2.1 Pioneers

3D bioprinting arose from the multidisciplinary amalgamation of several other relatively cutting-edge technologies—additive manufacturing and cell patterning. Additive manufacturing had been present for some time for other applications,

fabricating devices, components, and parts from materials such as metals and plastics. Cell patterning and substrate patterning were related technologies employed in research labs for applications such as probing cell–protein interactions. In the early 2000s, several researchers: Vladimir Mironov, Gabor Forgacs, and Thomas Boland, saw the natural combining of these technologies, and others, such as commercial inkjet printing, as a way to build 3D living structures that perhaps one day could serve as replacement tissues and organs in human patients.^{3,5,12} Thus was coined the term *organ printing*, the precursor to *bioprinting*, which is the term we have more broadly settled on.

Bioprinting hardware has evolved significantly (Figure 1.1). Early bioprinters were often custom built, hacked, and inkjet printers.^{13,14} The few labs working in these areas built their hardware themselves, and these custom devices were often incredibly difficult to operate, full of software bugs, and featured impossible user interfaces. Those lucky enough to receive substantial funding could utilize other 3D-printing devices that were commercially available, but these devices were not engineered to print biological materials, and ran U.S.\$100,000 to U.S.\$200,000 for a single operational piece of hardware.¹⁵ During this time, additive manufacturing continued to advance, particularly in the open-source world, resulting in a number of inexpensive, but still buggy, printers that were

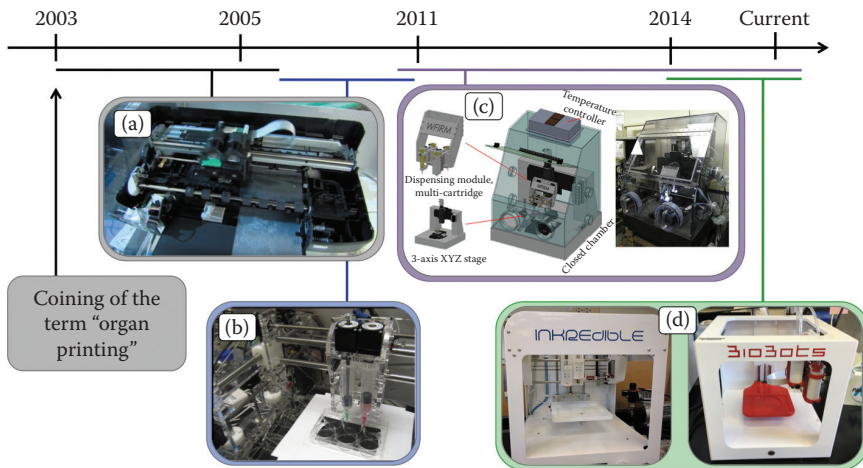


Figure 1.1 Evolution and examples of bioprinting hardware platforms. (a) A modified HP commercial inkjet printer for printing cells. (b) An open-source, Fab@Home "DIY" 3D printer employed for bioprinting purposes. (c) A custom-built 3D bioprinter with multinozzle capacity for printing hydrogels and melt-cure polymers. (d) Examples of relatively recent, commercially available bioprinters that are affordable for most biomedical research laboratories.

amenable to bioprinting, but only after substantial tinkering. These limitations made advancing of bioprinting technology difficult, but not impossible.

During this time, the technology continued to evolve and bifurcate, as did the terminology associated with other facets of bioprinting. Boland-led efforts continued to push inkjet-based bioprinting. In parallel, the Forgacs-led Frontiers in Integrated Biological Research (FIBR) effort built on biophysics concepts, that is, tissue liquidity and tissue fusion, developing a platform in which cell aggregates, or tissue spheroids, were deposited into a hydrogel biomaterial substrate, and based on both cell–cell and cell–matrix-based interactions would fuse in a controllable manner into larger bioengineered tissue constructs.^{14,16–21} Within this paradigm was coined the term *bioink*, referring to the cell aggregates. Likewise, the term *biopaper* was coined to reflect the hydrogel biomaterial component. Although over time, the term *biopaper* has all but disappeared as in the minds of most researchers, *bioink* encompasses cells, biomaterials, and combinations thereof.

1.2.2 Modalities defined

During this formative stage, the continued bifurcation of approaches occurred. The spheroid-in-hydrogel methodology evolved into two independent modalities. Cell aggregate bioprinting, and later cell–rod or cell–filament bioprinting techniques, became referred to as scaffold-free bioprinting, as this approach no longer relied on a biologically supportive hydrogel environment. Rather, inert materials such as agarose were used to physically support 3D arrays of cell aggregates and filaments while they formed into more complex structures through tissue fusion after which the agarose supports were removed. During the same period the materials that were developed as the biopaper to the cellular bioink were explored more directly as a bioink of sorts themselves, containing cells encapsulated within (Figure 1.2a). This approach, in which cells are generally encapsulated in a material, usually a hydrogel, and then printed from a syringe tip-like print head, is what is now referred to as extrusion bioprinting, and a number of labs and companies have their own bioink formulations.

1.2.2.1 Inkjet The first printing modality that we describe is inkjet bioprinting (Figure 1.2b), which can be further broken down into two approaches: (1) thermal and (2) piezoelectric. These two approaches have a similar overall methodology in which a printing cartridge or syringe is filled with bioink that is then forced through an output aperture. Thermal inkjet bioprinters are originally derived from the technology used in commercially available desktop inkjet printers, as we have described in the evolution of bioprinting earlier. In early instances of this approach, commercially available ink cartridges served as the print heads. The ink from these cartridges were removed and replaced with a bioink.^{22,23} Thermal inkjets

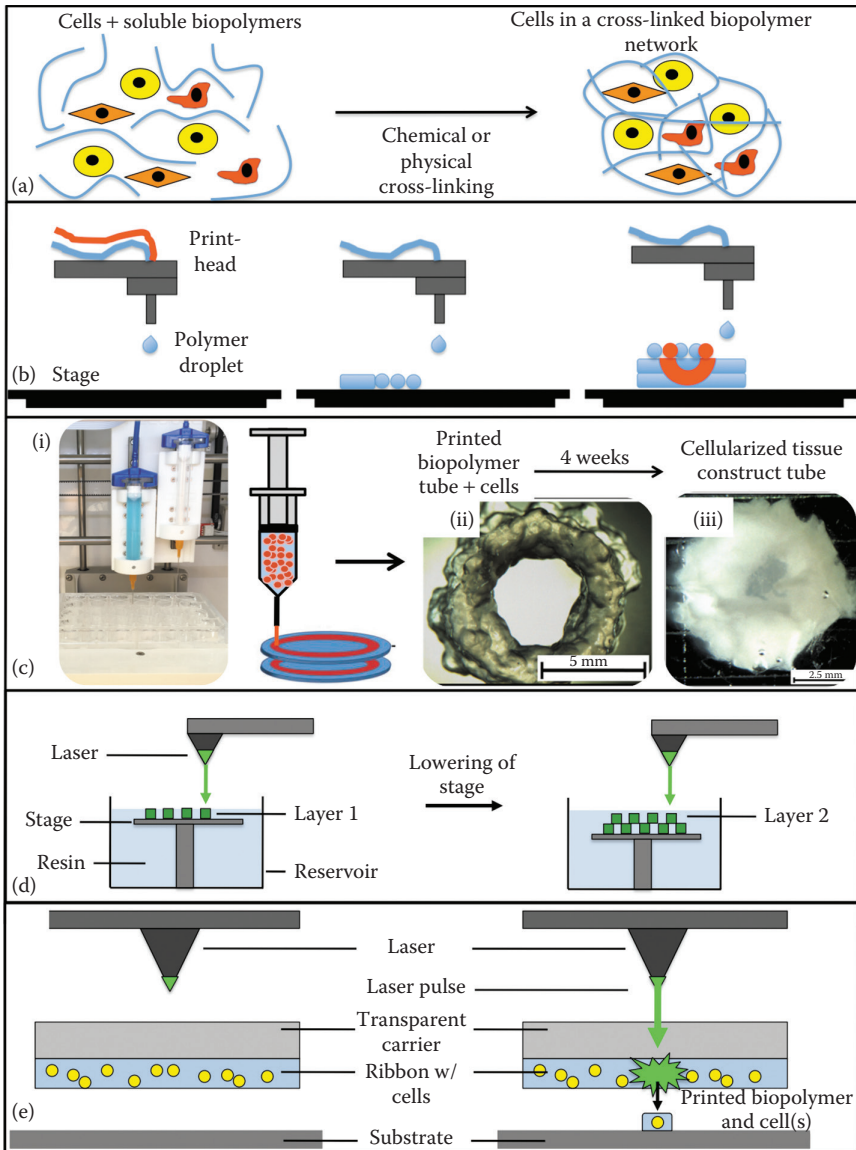


Figure 1.2 Bioprinting hardware modalities. (a) Prior and/or during bioprinting, cells are encapsulated within bioink biomaterials. Schematics describing (b) inkjet bioprinting, (c) extrusion bioprinting (i - an extrusion bioprinter; ii - a tubular construct immediately after printing; iii - over time the tubular construct is remodeled by the cells within into living tissue.), (d) stereolithography, and (e) laser-induced forward transfer (LIFT) bioprinting.

then use a heating element that creates a bubble in the bioink, generating pressure that forces the bioink through the output aperture of the print head. The specific parameters of these print heads are very much dependent on the particular type of cartridge used. Spatial resolution, in particular, is dependent on the type of inkjet printer that is being used, the size of the output aperture, and burst level and temperature driven by the thermal unit. In addition, spatial precision can be adjusted by altering the composition and properties of the bioink, such as concentration and viscosity, in relation to the average drop volume of the device.

Throughput in inkjet bioprinters can vary widely, and these printers with small output apertures have a tendency to rapidly clog if printing parameters or bioink properties are not optimal. Some groups have experimented with adding chemical agents to bioink formulations in an effort to alleviate clogging,²⁴ but this needs careful consideration to ensure that these supplements do not cause cell toxicity. Low overall throughput is another concern that prevents thermal inkjet printing from being used more widely for bioprinting applications. Small droplet volumes, and a lack of continuous deposition, results in increased printing times when biofabricating 3D constructs of significant size. Cell viability can also be a concern because of the thermodynamic properties of thermal inkjet printing. The thermal element in the cartridge briefly heats the ink to 300°C, which raises the temperature of the bioink by approximately 4°C–10°C.²⁵ Cell survival has been found to range from 70%–90% in most cases, although some groups have determined that certain cell types require a recovery period after bioprinting to restore membrane integrity and prevent cell death.^{25–28} Thermal inkjet printers are useful for their low cost and ease of customization; however, the issues with spatial resolution and throughput are significant limitations.

In contrast to thermal inkjet printing, piezoelectric inkjet printers do not alter the temperature of the bioink to create pressure inside the print cartridge. Piezoelectric printers use either acoustic waves or a physical component that changes shape or size to generate pressure changes inside the cartridge, which in turn forces the bioink through the output aperture.^{29,30} The acoustic wave-based approach, in particular, is highly controllable through the manipulation of the wave parameters including duration, amplitude, and frequency. Therefore, while spatial resolution can be variable, printing parameters are actually more controllable than in most thermal inkjet printers. Printing precision is generally excellent in piezoelectric inkjet printers because of the ability to control bioink droplet sizes. Throughput is also improved—these systems can be modified to remove the output nozzle, thus avoiding the issue of clogging that is prevalent with thermal inkjet printers. Cell viability has been observed in the range of 80%–90% in studies of encapsulated cells delivered through acoustic bioprinting.³¹

Even though piezoelectric inkjet bioprinting has several advantages over thermal inkjets for bioprinting, both these inkjet printing approaches suffer from

limitations with respect to bioinks.^{32,33} The greatest limitation is the viscosity of the bioink during printing. To form a small droplet, the bioink must be liquid inside the cartridge and rapid transition to a solid once it has exited the print head. This transformation is achieved by inducing cross-linking or gelation of the bioink chemically, enzymatically, physically, or in some other manner. Practically, this limitation means that bioinks for inkjet bioprinting are constrained to a maximum viscosity.³⁴ Moreover, the gelation requirement to form a solid or semisolid gel nearly instantaneously also places a limitation on materials that can be employed and chemical reactions that are feasible. Gelation agents typically form cross-links between molecules of the matrix and can range from chemical reactions to ultraviolet radiation to pH changes. However, some gelation agents are toxic to cells, which further limits the available bioinks.³⁵

1.2.2.2 Extrusion Extrusion-based deposition (Figure 1.2c), generally from syringe-like pieces of equipment housed on XYZ-mobile components in the bioprinter, is an additional approach for 3D bioprinting that primarily relies on controlling the mechanical and temporal characteristics of the materials being printed. In this bioprinting modality, the properties of the printed polymer or hydrogel facilitate extrusion through a syringe tip, commonly driven by pneumatic pressure or mechanical pistons controlled by the computer software that drives the printing protocol. The reliance on the material properties for printing means that the bioink not only must be soft or nearly fluid like enough to facilitate smooth extrusion through the small diameter tip or nozzle but must also be able to support itself mechanically in a 3D structure after deposition. The rheological properties of the material can be tailored in such a manner that the bioinks have a high enough elastic modulus, or the elastic component of its mechanical properties (similar to stiffness), such that extruded filaments of the bioink maintain their shape. Simultaneously, the material may maintain a sufficient loss modulus, or the fluid-like component, such that extrusion is still feasible.^{36,37} One common approach is to employ melt-curable polymers such as polycaprolactone, which when heated can be deformed and printed at a high resolution but can cool down to a solid material quickly after deposition.^{38,39} These materials can be used to build rather large and intricate structures, capable of mimicking physiological architectures with a significant load-bearing capacity. However, melt-cure printing cannot be performed with cells integrated in or on the polymers because of the conditions required during the melt-cure process. Specifically, often high heat or toxic solvents are required to decrease the elastic modulus of the polymers for extrusion. As such, cells must be seeded at a later time, or other cell-supportive materials can be incorporated separately, such as hydrogels, where the cells are encapsulated in the printing process.³⁹ Printing with hydrogels via extrusion techniques can be difficult when working with materials that rely on time for gelation to occur. Mistiming the deposition process can result in either a structure that collapses because cross-links have not formed quick enough, or conversely,

clogging of the bioprinter print head as a result of polymerization that was too fast, occurring prior to deposition. However, numerous studies have implemented novel cross-linking chemistries, photopolymerization techniques, and methods to facilitate spatial and temporal control over bioink material properties that can contain encapsulated cells for printing 3D structures.^{36,37,40–43}

Another approach to extrusion-based printing is what has been termed as *scaffold-free* bioprinting, which was derived from the concepts of tissue liquidity and tissue fusion of adjacent multicellular components.²¹ In this approach, aggregates, filaments, or tissue fragments comprised primarily of cells that are bound to one another by cell–cell adhesions are printed in geometric patterns or shapes and allowed to fuse over time to form larger constructs.¹⁸ Multiple layers of aggregates or filaments can be printed into physiological structures, and after fusing together during a postprint maturation period, singular 3D structures remain. This approach was used to build branched vascular structures,²¹ and more recently nerve grafts.⁴⁴ Despite the term *scaffold-free*, it should be noted that this type of bioprinting often does in fact rely on biomaterials. Typically, the cell aggregates or cell filaments are either printed into a cell-free biopolymer substrate or additively stacked using space-holding biomaterials such as agarose or other cell-free hydrogels to preserve the appropriate structures during the tissue fusion and maturation processes. These placeholders are generally removed when the construct is sufficiently fused and possesses sufficient mechanical properties to support itself without the placeholders. The strength of this method lies within its high cell density, which allows for rapid fusion between discretely printed pieces. This method has been explored extensively and is the base technology for the commercial entity Organovo, one of the first and most successful bioprinting-based companies. In addition, our laboratory previously developed a hydrogel-based approach that mimicked this technique, by printing hydrogel and cell-hydrogel filaments that fused over time to create tubular constructs *in vitro*,⁴⁵ and Bertassoni et al. used this approach to print HepG2 liver cells within methacrylated gelatin filaments.⁴⁶

1.2.2.3 Stereolithography and projection patterning Stereolithography (SL) is a long-used solid free-form fabrication technique that employs a reservoir containing photocurable polymer solution or resin, a laser with X–Y control, and a stage or fabrication platform with Z-axis control (Figure 1.2d).⁴⁷ Fabrication occurs at the surface of the reservoir and the stage lowers incrementally, allowing layers to be polymerized on top of each other, thus creating 3D structures in a bottom–up manner. There also exists a top–down SL approach, which is less common, but is employed for some applications. Resolution is modulated by the focus and energy of the laser, and as such has the capacity for very high resolution. Traditionally, SL has been used to create cell-free scaffolds and other structures not employed within tissue engineering (TE). However, with the development of biocompatible

polymers and protein systems with bioactive and cell-adherent properties that can be photopolymerized into hydrogels and scaffolds on demand, the potential for SL to be employed for TE applications has dramatically increased. Examples of biomaterials that are compatible with this technique are materials functionalized with acrylate, methacrylate, alkyne, and acrylamide groups, including gelatin–methacrylate, hyaluronic acid–methacrylate, polyethylene glycol diacrylate (PEGDA), polyethylene glycol diacrylamide, and polyethylene glycol dimethacrylate (PEGDMA).^{48–50} Recent developments have also led to projection SL that uses visible light as a curing source for cell-laden materials, thus minimizing the potential for cell damage from UV light sources and lasers.⁵¹ These techniques have also been multiplexed using digital mirror devices, which allows UV light to be applied to polymer solutions as projections of millions of individual points or pixels at once. This facilitates curing of entire layers of the 3D construct that is being formed at one time, greatly increasing the fabrication speed.⁵² This approach was recently employed to fabricate PEGDA-based liver architecture-inspired microdevices.⁵³

1.2.2.4 Laser-induced forward transfer Laser-induced forward transfer (LIFT)-based bioprinting (Figure 1.2e) is a laser-driven approach to bioprinting that has been adopted from other manufacturing and fabrication fields.^{54,55} LIFT technology was originally developed for high-resolution deposition and patterning of metals and other nonbiological materials for use in areas such as computer chip and component fabrication. More recently, it has been employed to create micro- and nanopatterned peptide, DNA, and cell arrays for running biological assays. LIFT technology is driven by using a laser that is pulsed in single bursts at desired time periods to correlate with deposition events and a donor *ribbon* materials comprised of the printable material, or bioink. The ribbon is supported on a transport substrate such as gold or titanium that will absorb the energy of the laser, after which it transfers the energy to the ribbon. When the energy pulse reaches the ribbon, a high-pressure bubble is generated that propels a droplet of the printable donor material onto a deposition stage below. Patterning is achieved by moving the stage, or alternatively the laser.^{56–58} For LIFT-based bioprinting, ribbons have been developed that comprise a biopolymer or protein, often similar to the materials used in the bioinks of other printing modalities. In LIFT bioprinting, the laser pulse-driven ribbon droplets contain cells, which are then deposited in a pattern on the movable stage to create cellular architectures and patterns. The lack of a nozzle in LIFT is a departure from other printing modalities, and generally does away with the need to prevent clogging issues. This results in an increased flexibility in the bioink material compositions, as long as they can be sufficiently transferred by the laser energy pulse. Studies have shown few negative effects on cell viability,^{59–61} and the ability to print nearly at a single cell per droplet resolution,⁶² positioning LIFT bioprinting as a technology with immense potential in the future for intricate tissue-engineering applications.

The high resolution of LIFT bioprinting is directed by a number of variables, such as the laser itself, the material properties of the printable bioink ribbon material, the relative hydrophilic/hydrophobic nature of the stage material to the printable bioink, cell density, and the path distance between the bioink ribbon and the stage.⁶³ Subsequently, there are also some challenges in LIFT bioprinting, which need to be overcome. The high resolution and subsequently small printing volume per pulse require fast gelation kinetics of the printable bioink material and a fast-moving stage for fabrication. In current LIFT protocols, preparation times of the ribbon, especially when containing cells and thus cell-compatible biomaterials, can be time-consuming. Furthermore, to create structures of size, multiple ribbons are often employed, requiring reloading during the printing process, which can be tedious. Nevertheless, with the potential to reach single-cell positioning accuracy, LIFT bioprinting holds great promise for creating intricate bioinspired architectures and tissue constructs.

1.2.3 Wider adoption

For years, we have seen an incredible growth in interest in bioprinting both in the research community and the general public ([Figure 1.1](#)). Perhaps the most important advancement in the field to date is the reduction in the cost of bioprinting hardware. In the past, commercially available 3D printers ran U.S.\$100,000 to U.S.\$200,000. Organovo, based on the Forgacs cell aggregate and filament technology, began their business of selling bioprinters to companies or large labs with significant resources—one NovoMax bioprinter ran approximately U.S.\$150,000. The custom-built Wake Forest Institute for Regenerative Medicine bioprinters of TedTalk fame ran about U.S.\$250,000 per platform. While hardware platforms at this cost level remain, there exists a collection of smaller companies now that sell bioprinters with small footprints for approximately U.S.\$5,000 to U.S.\$30,000, making it possible for smaller companies and academic labs with *average* funding levels to relatively easily afford bioprinters and integrate these technologies into their research.

1.3 Essential components of bioprinting

A number of bioprinting approaches have been recently explored, encompassing use of inkjet-like printers, extrusion devices, and laser-assisted devices. Regardless of the specific modality employed for bioprinting, there are three general variables that need to be thoroughly considered to successfully bioprint viable and functional tissue constructs. First is the cellular component comprising the living portion of the construct, which can be comprised of one cell type, but more often than not requires a complex but elegant interplay between multiple cell types to achieve significant tissue function. Second is the inclusion of supportive biomaterials, generally in the form of proteins and polymers that (1) facilitate the deposition method by mechanical means and (2) provide support

and protection to the cells during and after the tissue construct fabrication process. These biomaterials can encompass both the physical environment inside of which the cells will reside and the biochemical signal cells that need to function as they would in the body. Third is the actual bioprinting or biofabrication device itself and associated method by which fabrication is performed, be it inkjet, extrusion printing, or another modality. The way that these three variables are integrated plays the largest role in successful bioprinting. Most important, the biomaterial *bioink* employed serves as an essential bridge between the cells and the hardware. Unfortunately, across the field of bioprinting, less attention has been paid toward the development of bioprinting-specific biomaterials, and the fundamental chemical and material properties necessary for successful bioprinting. Although the hardware platforms have been described in detail earlier, in the following sections we describe the cellular and biomaterial-based components in more detail.

1.3.1 Cells

The cellular component in bioprinting is, of course, just as important a component, if not more, than the bioprinter hardware itself. In fact, the type, number, and combination of cells to be printed are often the first and foremost consideration to enter the minds of most researchers preparing to employ bioprinting in TE. This is rather to be expected, as in almost all cases, researchers have a tissue of interest in mind for which bioprinting is a platform technology that will be employed to create a tissue construct of that type. As such, it is natural that the first building blocks that are considered would be the cells and combinations of cells that can potentially give the bioprinted tissue its primary functional output.

Today, with the ever accelerating advances in cell biology and cell culture, we have numerous options for cell sources at our disposal that can be implemented for TE and bioprinting. These include established cell lines, primary cell lines taken from animal or human cadaver and donor tissues, and stem cells and cells derived from those stem cells that have been differentiated into functional cells. Furthermore, a substantial number of these cell sources are now commercially available to most laboratories and their implementation has been documented for a variety of applications, including tissue and tumor models for drug screening,^{9,64–70} disease modeling,^{71–74} stem cell therapy,⁷⁵ and organ-on-a-chip systems.^{9,41,76–78} Fortunately, we now have a significant repertoire of cell types from a variety of sources that can be employed in bioprinting tissue and organ construct protocols. However, each specific cell population can come with specific limitations in addition to their positive features.

1.3.1.1 Cell lines Cell lines are transformed cell populations that have gained the ability to divide for an indefinite period of time, resulting in fantastic sources of cells for cell biology laboratories. This gain in expansion potential is typically

because of immortalization in the laboratory by genetic manipulation or because the cell line is derived from a tumorigenic source. Established cell lines have been invaluable in research and have been employed in countless studies that have led to numerous important discoveries in the biomedical sciences. They are robust in nature, requiring relatively simple conditions and tissue culture practices to maintain. As such, these cell types are optimal for proof-of-concept work such as early stage development and testing of bioprinting hardware platforms and protocols. However, while cell lines do retain some of the normal functionality of the primary cells they are derived from, this functionality is often significantly diminished. Therefore, to construct truly functional bioprinted constructs that can accurately replicate or replace human tissues, other cell sources need to be considered.

1.3.1.2 Primary cells Primary cells specifically refer to cells that are isolated or harvested directly from living or recently living tissue or organs. With the exception of stem cell and progenitor populations, the majority of primary cells are terminally differentiated cells that have a highly specified functionality. Ideally, these cells would be the optimal cell populations for bioprinting 3D tissue constructs or organs. For example, primary liver hepatocytes are vastly superior to hepatocyte-derived cell lines such as HEPG2 and HEPG2 C3A in terms of secretion of biological compounds such as albumin and urea, as well as the ability to metabolize drugs and toxins.^{41,66,73} However, as most primary cells are terminally differentiated, they possess limited to no proliferative capacity and are sensitive to *in vitro* conditions experienced during standard 2D tissue cultures. Instead, they often require highly customized conditions or expensive tissue-specific tissue culture media formulations.

1.3.1.3 Stem cells and stem cell-derived cells Stem cells are defined by their ability to self-renew and produce progeny that can differentiate into specific functional cells.^{79–82} Stem cells are typically broken down into four types. Embryonic stem cells (ESCs) are derived from the inner cell mass of an embryo and are considered pluripotent—the ability to differentiate into all three germ layers. Second, adult stem cells exist in most tissues in the body and are generally constricted to differentiation into only the cell types that reside in that tissue. Third, perinatal stem cells (PSCs), which can be isolated from perinatal environments such as the placenta, umbilical cord, and amniotic fluid, are often considered to have plasticity somewhere between ESCs and adult stem cells. Finally, laboratory-generated, induced pluripotent stem (iPS) cells result from reprogramming terminally differentiated cells. Each of these stem cell types has advantages and disadvantages in TE and bioprinting applications. ESCs are attractive for their ability to differentiate into potentially any other cell type, thus, in theory, being able to repopulate any tissue in the human body. However, there are ethical concerns associated with procurement of ESCs, and on transplantation, they often form teratomas, thereby drastically limiting their clinical use. Adult stem

cells such as bone marrow-derived mesenchymal cells (BM-MSCs) are currently being used clinically for a small set of indications and are continuing to work their way through the regulatory pathways for a variety of applications,^{83,84} but their use is often restricted by limited proliferative capacity *in vitro*. Moreover, there is an inherent limit on differentiation potential MSCs placed on them by already being partially differentiated. iPS cells are less well characterized but have been shown to sometimes form teratomas after transplantation.⁸⁵ Our laboratory has extensively explored the use of PSCs, particularly those isolated from the amniotic fluid. However, they can be isolated from additional sources, such as amniotic membrane, placenta, umbilical cord, and Wharton's jelly.⁸⁶ Unlike ESCs, FSCs carry with them no ethical concerns because their isolation does not put the developing fetus at risk. Since cells in the extraembryonic environment arise early during pregnancy may give them with a higher degree of plasticity than adult stem cells, such as BM-MSCs or adipose-derived stem cells.⁷⁹ Most importantly, like MSCs, amniotic fluid-derived stem (AFS) cells, and likely other FSCs, secrete a wide range of trophic factors that are immunomodulatory and promote regeneration.^{75,87,88} Moreover, cells and biological compounds from the perinatal environment have recently been shown to have beneficial effects in regenerative medicine-based technologies such as wound healing and skin regeneration.^{43,75,89}

As described earlier, differentiation potential among stem cell types varies greatly. However, differentiated cells from stem cell populations are a potentially optimal source for bioprinting and TE applications. Differentiated cells may be able to more sufficiently recapitulate the level of function of corresponding primary cells than cell lines can. However, it should be noted that in most cases, cells differentiated in the laboratory are still not at the same level in terms of tissue-specific functionality as cells actually derived from viable tissues. Nevertheless, as some stem cell varieties possess heightened proliferative capacity, they theoretically can be expanded to extraordinary number prior to differentiation, thereby providing a nearly limitless source of relatively highly functioning cells.

1.3.2 Biomaterials

Biomaterials have an integral role in interfacing between hardware and biology. The materials employed for bioprinting can vary dramatically depending on the printing hardware that is used. These materials fall within the general category of biomaterials, as they often integrate and even support biological components such as cells, growth factors, or cytokines, and the actual *in vivo* wound environments. The term biomaterials itself comprises a massive range of materials that continues to evolve and be redefined, ranging from cell-supportive malleable hydrogels to stiff and durable metal or ceramic implants, from nanometer-sized nanoparticles for drug delivery and imaging to electronic medical devices such as

pacemakers, hearing aids, and artificial hearts. As development in materials science and biological exploration continues, so will the number classifications and definitions of biomaterials.^{90,91}

Only a subset of the entire biomaterials portfolio is suitable to act as bioinks for bioprinting. In the overall arena of bioprinting and biofabrication, biomaterials are generally confined to one of two primary categories. The first is that of melt-curable polymers that result in mechanically durable materials that act as scaffolding in tissue or cellularized constructs. These are generally employed in 3D construct fabrication rather than bioprinting of skin. Many such materials typically require high temperatures or toxic solvents to facilitate dissolution and shaping of the base polymers, and therefore are usually not appropriate for printing together with cells or other sensitive biological agents. Instead, these scaffolds are often formed first, after which cells are seeded onto and into the scaffolds after fabrication. The second category of biomaterials is that of soft and malleable biomaterials such as hydrogels, which generally has a high aqueous content, inside of which cells are capable of residing while remaining viable. These can be comprised of synthetic or natural polymers and in most of the cases, these do not possess the same levels of mechanical properties (stiffness, tensile strength, elastic modulus, etc.) as curable polymers in mechanical support roles. The inherent characteristics of these different printing materials, including mechanical properties, melting points, and available chemistries for cross-linking and functionalization, are responsible for determining whether or not one can successfully use them for bioprinting. Fortunately, these different parameters can be harnessed and manipulated to yield a virtually limitless toolbox of materials and bioink formulations for bioprinting, as well as other biofabrication approaches.

A common hurdle that many researchers working at the nexus of biomaterials and bioprinting face is that most biomaterials were not originally designed specifically for bioprinting applications. To overcome this limitation, much of the work in the early and current bioprinting has focused on adapting more traditional materials through chemical and mechanical manipulations to better integrate with bioprinting hardware, while maintaining cell-friendly and cell-supportive characteristics. To be considered such, these materials must not result in toxicity in cells and optimally should provide cell-adherence motifs, such as RGD (arginylglycylaspartic acid) peptides, to allow for cell attachment and migration. Techniques to generate cross-linking reactions can be designed and implemented to be noncytotoxic, allowing efficient encapsulation of various cell types within the hydrogel matrices at the time of printing. This has become an important characteristic as there has been a movement in biomedical research from traditional 2D-cell culture platforms to 3D systems that better mimic *in vivo* conditions.⁹² All *in vivo* tissues are 3D in nature, and bioprinting should echo this characteristic, employing a 3D approach.⁹³

Hydrogel bioink materials fall into one of two additional subcategories: (1) synthetic hydrogels, which are comprised of polymers that are synthesized in the laboratory or (2) naturally derived hydrogels, which are comprised of polymers, often polysaccharides, but can also be comprised of peptides or proteins, purified from natural sources, and are often further manipulated in the laboratory. Common examples of synthetic hydrogels include polyethylene glycol (PEG)-based materials, such as PEGDA and polyacrylamide (PAAm)-based gels. Examples of naturally derived hydrogels that are in common use and have been explored for bioprinting include collagen, hyaluronic acid, gelatin, alginate, and fibrin. In general, synthetic materials allow for an increased control over molecular weights and distributions, as well as cross-linking chemistries, supporting precise control of mechanical properties such as elastic modulus E' and degradation kinetics. Conversely, naturally derived materials can be more difficult to modify to have specific physical properties, but often have innate bioactive characteristics via biological peptide sequences and cell attachment motifs that cells interact with, improving tissue integration and biocompatibility. However, it should be noted that this list is but an overview and is not exhaustive; many other materials exist that can be used in bioprinting or modified for bioprinting, and others are currently being developed.

As described earlier, we initially employed a fibrin–collagen bioink system to be used with the skin bioprinter developed by our group,⁹⁴ which employs pneumatic pressure and a series of valves in-line with different cartridges to deliver the bioink. Specifically, the fibrin-precursor fibrinogen was mixed with soluble collagen Type I giving the first part of a two-part system, whereas a solution of thrombin comprised the second part of the system. These solutions were then printed separately, only gelling on deposition after coming into contact with one another.^{75,95} Notably, the fibrinogen–thrombin gelation mechanism facilitated relatively fast cross-linking *in situ* and the collagen component cross-linked slowly within this protogel. While useful, we thought that this fibrin–collagen gel was not the most optimal material for this wound healing bioprinting. It required two solutions, thus relying on diffusion for mixing, therefore suffering from not polymerizing immediately *in situ*. In irregular 3D-wound environments or convex surfaces, this resulted in some sloughing off of the material before gelation was complete. Moreover, collagen often contracts during its gelation process, and the collagen Type I isoform is also the primary extracellular matrix (ECM) component in scarring, which we thought could potentially act directly against the healthy, nonscarred skin regeneration we were trying to attain. In light of this belief, we assessed a variety of other hydrogels commonly used in various regenerative medicine and TE application gelation times, ease of use, biocompatibility, immunogenicity, and bioprinter compatibility. As expected, the different materials exhibited differing pros and cons, again suggesting that a particular material should be chosen based on the particular target application. However, we identified additional

hydrogel formulations that were suitable for integration in our skin bioprinter and likely good choices for use as hydrogel bioinks in wound healing treatments.⁹⁶ One such hydrogel, a UV-photopolymerizable hyaluronic acid and gelatin material, capable of elastic modulus modulation, cytokine loading, and cell delivery, is currently being employed in a variety of applications in our laboratory, including skin printing.

1.4 Future hurdles and potential

1.4.1 Bioink limitations

Apart from bone and teeth, hydrogels allow for mimicry of the range of elastic modulus (E') values associated with nearly all other tissues of the body. Furthermore, processing techniques to generate sol–gel transitions can be designed to be noncytotoxic, allowing simple encapsulation of cells.^{97,98} This is important as there is an increasing movement from 2D to 3D cell and tissue culture in the fields of TE, regenerative medicine, and tissue modeling.⁹² Hydrogels that support encapsulation procedures are infinitely more efficient for 3D uses than rigid scaffold-seeding approaches of the past. One of the major problems that the field of bioprinting currently faces is the lack of biomaterials that are designed specifically for use in bioprinting. Much work has focused on adapting more traditional materials to bioprinting processes and hardware, instead of using bioprinting parameters as blueprints from which new materials are developed. This has become increasingly clear in recent years as the cost of bioprinting hardware has decreased, allowing more laboratories and companies to invest in bioprinting capabilities. However, many researchers fall back on traditional biomaterials with which they are familiar with, which were never designed to be compatible with the dynamic events encountered during bioprinting. As a result, there is a need for bioprintable biomaterials that are simple to implement in a wide variety of bioprinter hardware platforms.

While there do exist cross-linking strategies that work in bioprinting, these bioinks, despite being more nuanced than the majority of hydrogels available, need to be made more elegant and user-friendly for widespread adoption. For many bioinks, specific elastic modulus values are customized on the basis of hardware requirements. For example, a higher resolution print head (i.e., smaller needle tip) may require a softer bioink for smooth extrusion. This means customization for each different bioprinter. Instead, a bioink that can actually respond dynamically to the bioprinter itself would have more cross-platform compatibility.

Thixotropic properties may solve this problem.⁹⁹ A thixotropic hydrogel exists at some given G' until a certain level of mechanical stress is applied to the material. Then the material mechanically fails, and G' falls drastically, causing the material to behave as a viscous fluid. When the stress is removed, G' recovers and the material is once again a hydrogel. This behavior is described in [Figure 1.3a–c](#), in which

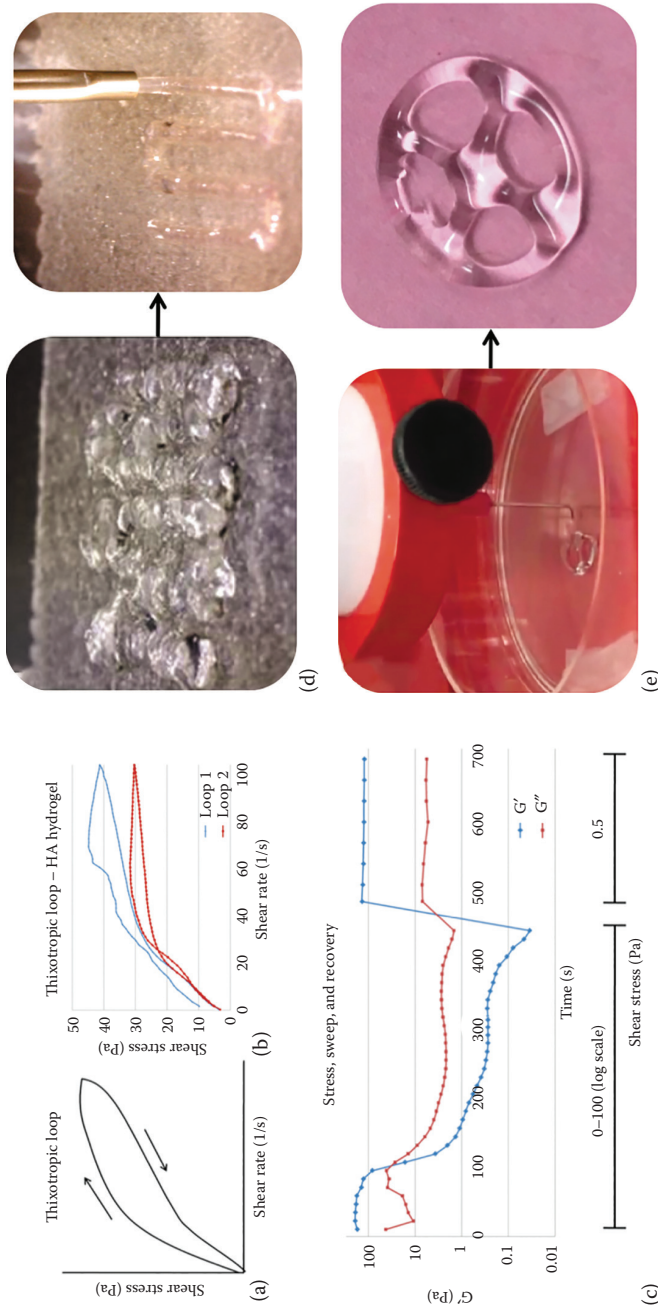


Figure 1.3 Harnessing mechanical properties to generate better bioinks for bioprinting. Thixotropy, similar to shear thinning, can improve bioink compatibility with bioprinters. (a, b) Thixotropic hysteresis loops—theoretical and measured by rheology in a hyaluronic acid-based hydrogel. (c) A stress sweep and recovery rheology protocol that indicates thixotropy in a hyaluronic acid-based hydrogel. (d, e) Images showing improvements in extrusion smoothness and bioprinting of 3D structures.

two rheological tests for thixotropy are shown—(1) the thixotropic hysteresis loop and (2) a stress relaxation and recovery test. These thixotropic properties lend themselves well to bioprinting. In the context of bioprinting, after being loaded into a bioprinter cartridge or syringe, the pressure during printing puts stress on the material as it is forced through the print head or needle tip. At this point, G' dynamically decreases, and the now viscous fluid, passes through the print head easily. On reaching the site of deposition, the stress is removed and the material returns to the original G' value in its 3D-printed form. This will ensure smooth and consistent extrusion, void of any clumping or fracturing of the biomaterial (Figure 1.3d–e). Development of novel biomaterials, engineered on the basis of the parameters and forces encountered during bioprinting protocols, has a huge potential to accelerate bioprinting toward viable commercial implementation.

1.4.2 Resolution versus speed

Throughout the development of bioprinting technology, the limitations of resolution have continued to be explored and pushed in a quest to bioprint more precise and more intricate 3D structures.^{100,101} High-resolution bioprinting, potentially at the single-cell level—which may be attainable soon using LIFT bioprinting—will likely be critical in bioprinting tissues with intricate architectures, particularly in cases where these architectures are crucial to tissue functions. Examples of this include architectures such as liver sinusoids and kidney nephrons, both structures where the architecture strongly governs tissue functionality.^{102,103} Reaching single-cell resolution—or close to it—will allow the biofabrication of these intricate structures by supporting the placement of individual cells in the exact positions they occupy *in vivo*. However, with increased resolution comes another limitation, that is, speed and time constraints for bioprinting. Specifically, as the unit volume of bioink decreases with increased resolution, it will require more units of bioink to biofabricate a construct of a particular size. Put plainly, printing at single-cell resolution will likely take an incredibly large amount of time to create a human-scale tissue construct. For example, in the human liver, 1 g of tissue contains roughly 140 million cells,¹⁰⁴ and a human liver weighs about 1.5 kg. This means that to print such a tissue at single-cell resolution, more than 210 billion individual single-cell depositions are required. This would be an unreasonable fabrication process, requiring an exorbitant amount of time. Moreover, during this time one would have to ensure that all of the cells that were still in bioink cartridges or print heads, or cells that had already been printed, are still viable. Given these limitations, it is more likely that single-cell resolution bioprinting will need to be paired with other modalities, such as extrusion printing, to simplify portions of a given tissue bioprinting protocol.

1.4.3 Regulatory hurdles

Strategies for cost-efficient, large-scale regenerative medicine clinical manufacturing must be developed that can generate these clinical products and ensure the economic viability of the clinical manufacturing industry. Bioprinting is expected to become a key element of regenerative medicine clinical manufacturing, and bioink formulations will need to be developed that are tailored for this purpose. However, significant regulatory hurdles exist. Tissue engineered or bioprinted tissues exist in a unique position with respect to how they are regulated by the Food and Drug Administration (FDA). Tissue products are inherently comprised of combinations of components that are considered drugs, biologicals, and devices. Due to this multifaceted classification, these products pose a difficult set of questions and requirements for the FDA and the researchers and developers who aim to bring these products to the market.^{105,106}

1.5 Conclusion

As viability, function, and safety concerns are demonstrated to be sufficient from a regulatory standpoint, demand for bioprinted tissue constructs as viable options for transplants in patients and screening tools for drug candidates will likely increase. To meet this eventual demand, not only will the supply of tissue-engineered constructs need to be expanded but so will the capabilities to maintain and mature the constructs, both of which can be aided by implementation of optimal biomaterials and environmental conditions. If successful bioprinting and biomaterial technologies advance as rapidly as they have in recent years, then perhaps *tissue engineering* (TE) of actual replacement tissues for diseased or damaged organs will become reality, potentially benefiting countless patients.

References

1. Visconti, R. P. et al. Towards organ printing: Engineering an intra-organ branched vascular tree. *Expert Opin Biol Ther* **10**, 409–420, doi:10.1517/14712590903563352 (2010).
2. Fedorovich, N. E. et al. Hydrogels as extracellular matrices for skeletal tissue engineering: State-of-the-art and novel application in organ printing. *Tissue Eng* **13**, 1905–1925, doi:10.1089/ten.2006.0175 (2007).
3. Mironov, V., Boland, T., Trusk, T., Forgacs, G. & Markwald, R. R. Organ printing: Computer-aided jet-based 3D tissue engineering. *Trends Biotechnol* **21**, 157–161, doi:10.1016/S0167-7799(03)00033-7 (2003).
4. Derby, B. Printing and prototyping of tissues and scaffolds. *Science* **338**, 921–926, doi:10.1126/science.1226340 (2012).
5. Boland, T., Mironov, V., Gutowska, A., Roth, E. A. & Markwald, R. R. Cell and organ printing 2: Fusion of cell aggregates in three-dimensional gels. *Anat Rec A Discov Mol Cell Evol Biol* **272**, 497–502, doi:10.1002/ar.a.10059 (2003).

6. Mironov, V., Kasyanov, V., Drake, C. & Markwald, R. R. Organ printing: Promises and challenges. *Regen Med* **3**, 93–103, doi:10.2217/17460751.3.1.93 (2008).
7. Norris, M. K. Organ transplantation: A crisis in supply, not demand. *Dimens Crit Care Nurs* **9**, 251–252 (1990).
8. Victoroff, M. S. The organ trail: Matching supply, demand. *Manag Care* **13**, 6–7 (2004).
9. Skardal, A. et al. Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Sci Rep* **7**, 8837 (2017).
10. OPTN: Organ Procurement and Transplantation Network Data Report, <http://optn.transplant.hrsa.gov/> (2010).
11. Thasler, W. E. et al. Human tissue for in vitro research as an alternative to animal experiments: A charitable “honest broker” model to fulfil ethical and legal regulations and to protect research participants. *Altern Lab Anim* **34**, 387–392 (2006).
12. Mironov, V. Printing technology to produce living tissue. *Expert Opin Biol Ther* **3**, 701–704 (2003).
13. Jakab, K., Neagu, A., Mironov, V. & Forgacs, G. Organ printing: Fiction or science. *Biorheology* **41**, 371–375 (2004).
14. Jakab, K., Neagu, A., Mironov, V., Markwald, R. R. & Forgacs, G. Engineering biological structures of prescribed shape using self-assembling multicellular systems. *Proc Natl Acad Sci U S A* **101**, 2864–2869 (2004).
15. Ozbolat, I. T., Moncal, K. K. & Gudapati, H. Evaluation of bioprinter technologies. *Additive Manufacturing* **13**, 179–200 (2017).
16. Fleming, P. A. et al. Fusion of uniluminal vascular spheroids: A model for assembly of blood vessels. *Dev Dyn* **239**, 398–406, doi:10.1002/dvdy.22161 (2010).
17. Jakab, K., Damon, B., Neagu, A., Kachurin, A. & Forgacs, G. Three-dimensional tissue constructs built by bioprinting. *Biorheology* **43**, 509–513 (2006).
18. Jakab, K. et al. Tissue engineering by self-assembly of cells printed into topologically defined structures. *Tissue Eng Part A* **14**, 413–421 (2008).
19. Mironov, V. et al. Organ printing: Tissue spheroids as building blocks. *Biomaterials* **30**, 2164–2174, doi:S0142-9612(09)00005-210.1016/j.biomaterials.2008.12.084 (2009).
20. Neagu, A., Jakab, K., Jamison, R. & Forgacs, G. Role of physical mechanisms in biological self-organization. *Phys Rev Lett* **95**, 178104 (2005).
21. Norotte, C., Marga, F. S., Niklason, L. E. & Forgacs, G. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials* **30**, 5910–5917, doi:S0142-9612(09)00640-110.1016/j.biomaterials.2009.06.034 [doi] (2009).
22. Boland, T., Xu, T., Damon, B. & Cui, X. Application of inkjet printing to tissue engineering. *Biotechnol J* **1**, 910–917 (2006).
23. Roth, E. A. et al. Inkjet printing for high-throughput cell patterning. *Biomaterials* **25**, 3707–3715 (2004).
24. Parzel, C. A., Pepper, M. E., Burg, T., Groff, R. E. & Burg, K. J. EDTA enhances high-throughput two-dimensional bioprinting by inhibiting salt scaling and cell aggregation at the nozzle surface. *J Tissue Eng Regen Med* **3**, 260–268 (2009).
25. Cui, X., Dean, D., Ruggeri, Z. M. & Boland, T. Cell damage evaluation of thermal inkjet printed Chinese hamster ovary cells. *Biotechnol Bioeng* **106**, 963–969 (2010).
26. Chang, R., Nam, J. & Sun, W. Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. *Tissue Eng Part A* **14**, 41–48 (2008).
27. Nair, K. et al. Characterization of cell viability during bioprinting processes. *Biotechnol J* **4**, 1168–1177 (2009).
28. Xu, T. et al. Viability and electrophysiology of neural cell structures generated by the inkjet printing method. *Biomaterials* **27**, 3580–3588 (2006).

29. Saunders, R. E., Gough, J. E. & Derby, B. Delivery of human fibroblast cells by piezo-electric drop-on-demand inkjet printing. *Biomaterials* **29**, 193–203 (2008).
30. Demirci, U. & Montesano, G. Single cell epitaxy by acoustic picolitre droplets. *Lab Chip* **7**, 1139–1145, doi:10.1039/b704965j (2007).
31. Tasoglu, S. & Demirci, U. Bioprinting for stem cell research. *Trends Biotechnol* **31**, 10–19, doi:10.1016/j.tibtech.2012.10.005 (2013).
32. Kim, J. D., Choi, J. S., Kim, B. S., Choi, Y. C. & Cho, Y. W. Piezoelectric inkjet printing of polymers: Stem cell patterning on polymer substrates. **51**, 2147–2154, doi:10.1016/j.polymer.2010.03.038 (2010).
33. Murphy, S. V. & Atala, A. 3D bioprinting of tissues and organs. *Nat Biotechnol* **32**, 773–785, doi:10.1038/nbt.2958 (2014).
34. Kim, J. D., Choi, J. S., Kim, B. S., Choi, Y. C. & Cho, Y. W. Piezoelectric inkjet printing of polymers: Stem cell patterning on polymer substrates. **51**, 2147–2154, doi:10.1016/j.polymer.2010.03.038 (2010).
35. Hennink, W. E. & van Nostrum, C. F. Novel crosslinking methods to design hydrogels. *Adv Drug Deliv Rev* **54**, 13–36 (2002).
36. Skardal, A., Zhang, J., McCoard, L., Oottamasathien, S. & Prestwich, G. D. Dynamically crosslinked gold nanoparticle–hyaluronan hydrogels. *Adv Mater* **22**, 4736–4740, doi:10.1002/adma.201001436 (2010).
37. Skardal, A. et al. Photocrosslinkable hyaluronan–gelatin hydrogels for two-step bioprinting. *Tissue Eng Part A* **16**, 2675–2685, doi:10.1089/ten.TEA.2009.0798 (2010).
38. Kang, H. W., Lee, S. J., Atala, A. & Yoo, J. J. Integrated organ and tissue printing methods, system and apparatus. United States of America patent US 2012/00889238 A1 (2011).
39. Kang, H. W. et al. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nat Biotechnol* **34**, 312–319, doi:10.1038/nbt.3413 (2016).
40. Pescosolido, L. et al. Hyaluronic acid and dextran-based semi-IPN hydrogels as biomaterials for bioprinting. *Biomacromolecules* **12**, 1831–1838, doi:10.1021/bm200178w (2011).
41. Skardal, A. et al. A hydrogel bioink toolkit for mimicking native tissue biochemical and mechanical properties in bioprinted tissue constructs. *Acta Biomater* **25**, 24–34, doi:10.1016/j.actbio.2015.07.030 (2015).
42. Skardal, A. et al. Bioprinting cellularized constructs using a tissue-specific hydrogel bioink. *J Vis Exp*, doi:10.3791/53606 (2016).
43. Skardal, A. et al. A tunable hydrogel system for long-term release of cell-secreted cytokines and bioprinted in situ wound cell delivery. *J Biomed Mater Res B Appl Biomater* **105**, 1729–2190, doi:10.1002/jbm.b.33736 (2016).
44. Marga, F. et al. Toward engineering functional organ modules by additive manufacturing. *Biofabrication* **4**, 022001, doi:10.1088/1758-5082/4/2/022001 (2012).
45. Skardal, A., Zhang, J. & Prestwich, G. D. Bioprinting vessel-like constructs using hyaluronan hydrogels crosslinked with tetrahedral polyethylene glycol tetracrylates. *Biomaterials* **31**, 6173–6181, doi:S0142-9612(10)00561-210.1016/j.biomaterials.2010.04.045 (2010).
46. Bertassoni, L. E. et al. Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels. *Biofabrication* **6**, 024105, doi:10.1088/1758-5082/6/2/024105 (2014).
47. Skoog, S. A., Goering, P. L. & Narayan, R. J. Stereolithography in tissue engineering. *J Mater Sci Mater Med* **25**, 845–856, doi:10.1007/s10856-013-5107-y (2014).
48. Billiet, T., Vandenhaute, M., Schelfhout, J., Van Vlierberghe, S. & Dubruel, P. A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials* **33**, 6020–6041, doi:10.1016/j.biomaterials.2012.04.050 (2012).
49. Soman, P., Chung, P. H., Zhang, A. P. & Chen, S. Digital microfabrication of user-defined 3D microstructures in cell-laden hydrogels. *Biotechnol Bioeng* **110**, 3038–3047, doi:10.1002/bit.24957 (2013).

50. Hribar, K. C., Soman, P., Warner, J., Chung, P. & Chen, S. Light-assisted direct-write of 3D functional biomaterials. *Lab Chip* **14**, 268–275, doi:10.1039/c3lc50634g (2014).
51. Lin, H. et al. Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. *Biomaterials* **34**, 331–339, doi:10.1016/j.biomaterials.2012.09.048 (2013).
52. Zhang, A. P. et al. Rapid fabrication of complex 3D extracellular microenvironments by dynamic optical projection stereolithography. *Adv Mater* **24**, 4266–4270, doi:10.1002/adma.201202024 (2012).
53. Gou, M. et al. Bio-inspired detoxification using 3D-printed hydrogel nanocomposites. *Nat Commun* **5**, 3774, doi:10.1038/ncomms4774 (2014).
54. Bohandy, J., Kim, B. & Adrian, F. Metal deposition from a supported metal film using an excimer laser. *J Appl Phys* **60**, 1538 (1986).
55. Barron, J. A., Ringeisen, B. R., Kim, H., Spargo, B. J. & Chrisey, D. B. Application of laser printing to mammalian cells. *Thin Solid Films* **453**, 383–387 (2004).
56. Chrisey, D. B. Materials processing: The power of direct writing. *Science* **289**, 879–881, doi:10.1126/science.289.5481.879 (2000).
57. Colina, M., Serra, P., Fernandez-Pradas, J. M., Sevilla, L. & Morenza, J. L. DNA deposition through laser induced forward transfer. *Biosens Bioelectron* **20**, 1638–1642, doi:10.1016/j.bios.2004.08.047 (2005).
58. Dinca, V. et al. Directed three-dimensional patterning of self-assembled peptide fibrils. *Nano Lett* **8**, 538–543, doi:10.1021/nl072798r (2008).
59. Hopp, B. et al. Survival and proliferative ability of various living cell types after laser-induced forward transfer. *Tissue Eng* **11**, 1817–1823, doi:10.1089/ten.2005.11.1817 (2005).
60. Gruene, M. et al. Laser printing of stem cells for biofabrication of scaffold-free autologous grafts. *Tissue Eng Part C Methods* **17**, 79–87, doi:10.1089/ten.TEC.2010.0359 (2010).
61. Koch, L. et al. Laser printing of skin cells and human stem cells. *Tissue Eng Part C Methods* **16**, 847–854, doi:10.1089/ten.TEC.2009.0397 (2010).
62. Guillotin, B. et al. Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials* **31**, 7250–7256, doi:10.1016/j.biomaterials.2010.05.055 (2010).
63. Guillemot, F., Souquet, A., Catros, S. & Guillotin, B. Laser-assisted cell printing: Principle, physical parameters versus cell fate and perspectives in tissue engineering. *Nanomedicine (Lond)* **5**, 507–515, doi:10.2217/nnm.10.14 (2010).
64. Skardal, A., Devarasetty, M., Soker, S. & Hall, A. R. In situ patterned micro 3D liver constructs for parallel toxicology testing in a fluidic device. *Biofabrication* **7**, 031001, doi:10.1088/1758-5090/7/3/031001 (2015).
65. Skardal, A., Shupe, T. & Atala, A. Organoid-on-a-chip and body-on-a-chip systems for drug screening and disease modeling. *Drug Discov Today* **21**, 1399–1411, doi:10.1016/j.drudis.2016.07.003 (2016).
66. Skardal, A. et al. Tissue specific synthetic ECM hydrogels for 3-D in vitro maintenance of hepatocyte function. *Biomaterials* **33**, 4565–4575, doi:S0142-9612(12)00321-310.1016/j.biomaterials.2012.03.034 (2012).
67. Benam, K. H. et al. Engineered in vitro disease models. *Annual Rev Pathol* **10**, 195–262, doi:10.1146/annurev-pathol-012414-040418 (2015).
68. Huh, D. et al. A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. *Sci Transl Med* **4**, 159ra147, doi:10.1126/scitranslmed.3004249 (2012).
69. Bhise, N. S. et al. Organ-on-a-chip platforms for studying drug delivery systems. *J Control Release* **190**, 82–93, doi:10.1016/j.jconrel.2014.05.004 (2014).

70. Polini, A. et al. Organs-on-a-chip: A new tool for drug discovery. *Expert Opin Drug Discov* **9**, 335–352, doi:10.1517/17460441.2014.886562 (2014).
71. Devarasetty, M., Skardal, A., Cowdrick, K., Marini, F. & Soker, S. Bioengineered sub-mucosal organoids for in vitro modeling of colorectal cancer. *Tissue Eng Part A* (2017).
72. Devarasetty, M., Wang, E., Soker, S. & Skardal, A. Mesenchymal stem cells support growth and organization of host-liver colorectal-tumor organoids and possibly resistance to chemotherapy. *Biofabrication* **9**, 021002, doi:10.1088/1758-5090/aa7484 (2017).
73. Skardal, A., Devarasetty, M., Forsythe, S., Atala, A. & Soker, S. A reductionist metastasis-on-a-chip platform for in vitro tumor progression modeling and drug screening. *Biotechnol Bioeng* **113**, 2020–2032, doi:10.1002/bit.25950 (2016).
74. Skardal, A., Devarasetty, M., Rodman, C., Atala, A. & Soker, S. Liver-tumor hybrid organoids for modeling tumor growth and drug response in vitro. *Ann Biomed Eng* **43**, 2361–2373, doi:10.1007/s10439-015-1298-3 (2015).
75. Skardal, A. et al. Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. *Stem Cells Transl Med* **1**, 792–802, doi:sctm.2012-008810.5966/sctm.2012-0088 (2012).
76. Devarasetty, M. et al. Optical tracking and digital quantification of beating behavior in bioengineered human cardiac organoids. *Biosensors (Basel)* **7**, doi:10.3390/bios7030024 (2017).
77. Zhang, Y. S. et al. Multisensor-integrated organs-on-chips platform for automated and continual in situ monitoring of organoid behaviors. *Proc Natl Acad Sci U S A* **114**, E2293–E2302, doi:10.1073/pnas.1612906114 (2017).
78. Zhang, Y. S. et al. Bioprinting 3D microfibrinous scaffolds for engineering endothelialized myocardium and heart-on-a-chip. *Biomaterials* **110**, 45–59, doi:10.1016/j.biomaterials.2016.09.003 (2016).
79. De Coppi, P. et al. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* **25**, 100–106, doi:nbt127410.1038/nbt1274 (2007).
80. Furth, M. E. & Atala, A. Stem cell sources to treat diabetes. *J Cell Biochem* **106**, 507–511, doi:10.1002/jcb.22000 (2009).
81. Joo, S., Ko, I. K., Atala, A., Yoo, J. J. & Lee, S. J. Amniotic fluid-derived stem cells in regenerative medicine research. *Arch Pharm Res* **35**, 271–280, doi:10.1007/s12272-012-0207-7 (2012).
82. Murphy, S. V. & Atala, A. Organ engineering—combining stem cells, biomaterials, and bioreactors to produce bioengineered organs for transplantation. *BioEssays* **35**, 163–172, doi:10.1002/bies.201200062 (2013).
83. Keating, A. Mesenchymal stromal cells: New directions. *Cell Stem Cell* **10**, 709–716, doi:S1934-5909(12)00249-410.1016/j.stem.2012.05.015 (2012).
84. Mulder, G. D., Lee, D. K. & Jeppesen, N. S. Comprehensive review of the clinical application of autologous mesenchymal stem cells in the treatment of chronic wounds and diabetic bone healing. *Int Wound J*, doi:10.1111/j.1742-481X.2011.00922.x (2012).
85. Fu, X. & Xu, Y. Challenges to the clinical application of pluripotent stem cells: Towards genomic and functional stability. *Genome Med* **4**, 55, doi:gm35410.1186/gm354 (2012).
86. Abdulrazzak, H., Moschidou, D., Jones, G. & Guillot, P. V. Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues. *J R Soc Interface* **7 Suppl 6**, S689–S706, doi:rsif.2010.0347.focus10.1098/rsif.2010.0347.focus (2010).
87. Moorefield, E. C. et al. Cloned, CD117 selected human amniotic fluid stem cells are capable of modulating the immune response. *PLoS One* **6**, e26535, doi:10.1371/journal.pone.0026535PONE-D-11-16225 (2011).
88. Caplan, A. I. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* **213**, 341–347, doi:10.1002/jcp.21200 (2007).

89. Murphy, S. V. et al. Solubilized amnion membrane hyaluronic acid hydrogel accelerates full-thickness wound healing. *Stem Cells Transl Med* **6**, 2020–2032, doi:10.1002/sctm.17-0053 (2017).
90. Williams, D. The continuing evolution of biomaterials. *Biomaterials* **32**, 1–2, doi:S0142-9612(10)01245-7/10.1016/j.biomaterials.2010.09.048 (2011).
91. Williams, D. F. On the nature of biomaterials. *Biomaterials* **30**, 5897–5909, doi:S0142-9612(09)00726-1/10.1016/j.biomaterials.2009.07.027 (2009).
92. Prestwich, G. D. Evaluating drug efficacy and toxicology in three dimensions: Using synthetic extracellular matrices in drug discovery. *Acc Chem Res* **41**, 139–148 (2008).
93. Mironov, V., Reis, N. & Derby, B. Review: Bioprinting: A beginning. *Tissue Eng* **12**, 631–634 (2006).
94. Yoo, J. J. et al. Delivery system. United States of America patent US 2011/0172611 A1 (2011).
95. Xu, T. et al. Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications. *Biofabrication* **5**, 015001, doi:10.1088/1758-5082/5/1/015001 (2013).
96. Murphy, S. V., Skardal, A. & Atala, A. Evaluation of hydrogels for bio-printing applications. *J Biomed Mater Res A* **101**, 272–284, doi:10.1002/jbm.a.34326 (2013).
97. Skardal, A. In *Essentials of 3D Biofabrication and Translation* A. Atala & J. J. Yoo (Eds.), London, UK: Elsevier, 2015.
98. Skardal, A. & Atala, A. Biomaterials for integration with 3-d bioprinting. *Ann Biomed Eng* **43**, 730–746, doi:10.1007/s10439-014-1207-1 (2015).
99. Pek, Y. S., Wan, A. C., Shekaran, A., Zhuo, L. & Ying, J. Y. A thixotropic nano-composite gel for three-dimensional cell culture. *Nat Nanotechnol* **3**, 671–675, doi:nnano.2008.27010.1038/nnano.2008.270 (2008).
100. Mir, T. A. & Nakamura, M. Three-dimensional bioprinting: Toward the era of manufacturing human organs as spare parts for healthcare and medicine. *Tissue Eng Part B Rev* **23**, 245–256, doi:10.1089/ten.TEB.2016.0398 (2017).
101. Hospodiuk, M., Dey, M., Sosnoski, D. & Ozbolat, I. T. The bioink: A comprehensive review on bioprintable materials. *Biotechnol Adv* **35**, 217–239, doi:10.1016/j.biotechadv.2016.12.006 (2017).
102. Kang, Y. B. et al. Liver sinusoid on a chip: Long-term layered co-culture of primary rat hepatocytes and endothelial cells in microfluidic platforms. *Biotechnol Bioeng* **112**, 2571–2582, doi:10.1002/bit.25659 (2015).
103. Marsh, D. J. et al. Architecture of the rat nephron-arterial network: Analysis with micro-computed tomography. *Am J Physiol Renal Physiol* **313**, F351–F360, doi:10.1152/ajprenal.00092.2017 (2017).
104. Sohlenius-Sternbeck, A. K. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicol In Vitro* **20**, 1582–1586, doi:10.1016/j.tiv.2006.06.003 (2006).
105. Patuzzo, S., Goracci, G., Gasperini, L. & Ciliberti, R. 3D bioprinting technology: Scientific aspects and ethical issues. *Sci Eng Ethics*, doi:10.1007/s11948-017-9918-y (2017).
106. Hourd, P., Medcalf, N., Segal, J. & Williams, D. J. A 3D bioprinting exemplar of the consequences of the regulatory requirements on customized processes. *Regen Med* **10**, 863–883, doi:10.2217/rme.15.52 (2015).

Chapter 2 Bioinks for 3D printing

E. Gargus, P. Lewis, and R. Shah

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2.1 Introduction

3D printing has received a significant amount of interest in the medical community from both academic and industrial settings. Within the past decade, the commercialization of a variety of 3D-printing platforms has decreased cost and enabled researchers to expand 3D-printing platforms to numerous applications.

One of the more exciting applications of 3D printing in medicine is within the field of tissue engineering (TE). The traditional TE paradigm combines a scaffold with signaling factors and cells seeded on top (Langer and Vacanti 2016). This approach has, and continues to, sustain the majority of TE approaches in both academic and industrial settings, and it certainly holds promise for engineering thin and/or tubular tissues such as the epidermis or trachea (Jin et al. 2011; Kojima and Vacanti 2014). The approach to engineering more complex tissues using top seeding has involved heterogeneous mixtures of cells within traditionally fabricated scaffolds using methods such as electrospinning, freeze casting, salt leaching, or gas foaming. Although some control over tissue formation can be exerted by modifying processing parameters to change pore size, microfiber diameter, and pore interconnectivity, 3D printing emerged in the late 1990s as a method to produce scaffolds with a significantly greater degree of architectural control that had not been previously demonstrated. 3D printing makes mass production of patient-specific scaffolds feasible. Researchers now have the ability to precisely control the spatial organization of both biomaterials and cells in one construct to create more sophisticated and biomimetic engineered tissue structures.

Bioprinting is defined as the patterning of cell-encapsulating biomaterials (bioinks) in three dimensions. The only class of materials capable of maintaining cell viability during encapsulation is hydrogels. Hydrogels by definition are polymer networks that hold more than 90% water, allowing for sufficient diffusion of nutrients and waste to support viability of encapsulated cells (Lee and Mooney 2001). They are also the closest approximation to the native extracellular matrix (ECM) available to tissue engineers. In this chapter, we will discuss the current state of the art of the available bioinks. Although we will briefly touch on other bioprinting methods, this chapter will primarily focus on extrusion-based bioink printing methodologies and formulations as they are the most versatile and well-studied for TE applications.

2.2 The need for tunable bioinks

To date, there is a limited palette of available 3D-printable bioinks that adequately represents the biological, chemical, and mechanical diversity seen in tissues and organs. Moreover, there is a limited ability to tune bioink material properties without compromising printability. In large part, this lack of tunability is because of the fact that qualities that make an ideal 3D-printable ink are at odds with the material properties that support high cell viability and function as illustrated in [Figure 2.1](#). For example, inks with high polymer concentrations are the most amenable to 3D printing, but cell viability is best at low polymer concentrations. To advance bioprinting, there is an urgent need to develop new tunable bioinks and bioprinting strategies for optimal biomaterial printability, as well as cell viability and function.

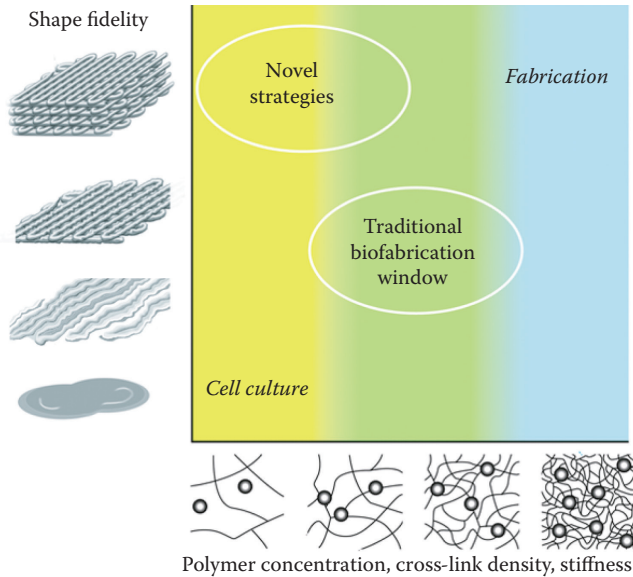


Figure 2.1 Additive manufacturing typically utilizes materials with high stiffness, cross-link density, and polymer concentration to maintain good shape fidelity; however, this is usually at the expense of cell viability and function. (From Malda, J. et al.: 25th Anniversary Article: Engineering Hydrogels for Biofabrication. *Advanced Materials*. 2013. 25. 5011–5028. Copyright John Wiley & Sons. Reprinted with permission.)

2.3 Liquid and gel-phase bioinks

As mentioned previously, the material properties of an ideal bioink are at odds with the material properties of an ideal cell-encapsulating gel (Figure 2.1). In addition, the act of extrusion imparts a large amount of stress on cells encapsulated within a bioink. Some researchers have taken advantage of liquid-phase bioinks, which dramatically reduce the shear stress that the cells are subjected to on extrusion. Liquid-phase bioinks, however, cannot easily be printed into multilayered structures alone and usually require a support structure or a sacrificial material and/or a secondary cross-linking method. Liquid-phase bioinks may also present issues with cell settling (Pepper et al. 2012). Periodic stirring has shown to keep cells in a homogenous suspension during inkjet printing, though continuous stirring can have negative effects on the cell viability (Parsa et al. 2010; Ferris et al. 2013). More viscous inks can slow the cell-settling process, leading to more homogenous printed constructs if structures are printed in a timely manner. In contrast, gel-phase bioinks allow for a number of advantages, including a lack of cell settling and higher shape fidelity (Rutz et al. 2015; Dubbin et al. 2016). They also have the ability to be self-supporting, and do not require sacrificial support materials to be co-printed.

2.4 Bioink cross-linking

Cross-linking is the step that converts polymer solutions into hydrogels. Cross-linking may take place before, during, or after 3D bioprinting. Due to the presence of cells, it is critical to consider the cytocompatibility of cross-linking reactions used for bioinks. In general, cross-linking can be categorized as physical or chemical. Physical cross-links are formed by ionic bonding, hydrogen bonding, hydrophobic forces, and molecular entanglements. Physical cross-links are reversible. While this reversibility allows physically cross-linked bioinks to be extruded in a cross-linked state, it can be problematic for its end application. For example, ionic cross-linking is vulnerable to dissolution in culture or *in vivo* where local divalent cation concentrations may draw cross-linking calcium out of the gel. On the other hand, chemical cross-links are formed by covalent bonds between polymer chains. Chemical cross-links are permanent, so fully chemically cross-linked bioinks usually cannot be 3D-printed because they are too robust for extrusion through fine-diameter nozzles. To overcome this challenge, some researchers extrude lightly cross-linked bioinks and enhance the mechanical properties post-printing with a secondary cross-linking step. It should be noted that a bioink can be formed through a combination of physical and chemical cross-linking.

Photopolymerization is a specific type of chemical cross-linking that is activated by light. Synthetic polymers are typically used; however, natural polymers can also be functionalized with acrylate groups to enable photopolymerization. On UV exposure, either with or without a photoinitiator, acrylates will undergo rapid free-radical polymerization (Lee et al. 2003). Tsang et al. used this approach to pattern 3D lobule-like structures using polyethylene glycol diacrylate (PEGDA) (Liu Tsang et al. 2007). UV-induced photopolymerization is an attractive secondary stabilization mechanism for extrusion-based 3D-bioprinting systems, as a material can be exposed to UV directly after extrusion or in a layer-by-layer fashion, fabricating a self-supporting structure. This strategy does, however, lead to cumulative UV exposure of initial printed layers, which may affect cell viability. In addition, a recurring and significant drawback to photopolymerized hydrogels, both natural and synthetic, is the toxic nature of photoinitiators necessary for the reaction to occur. Billiet et al. (2014), however, demonstrated that certain photoinitiators are more cytocompatible. For example, VA-086 led to less of a reduction in cell viability compared to the traditionally used Irgacure 2959 (2014).

2.5 Important considerations for bioprinting

There are also several other factors that are important to consider when designing bioinks for TE applications that have many interdependent relationships, as shown in [Figure 2.2](#). First, to mimic tissues and organs, it may be important to design tissue-specific inks, with mechanical properties and biochemical cues specific to

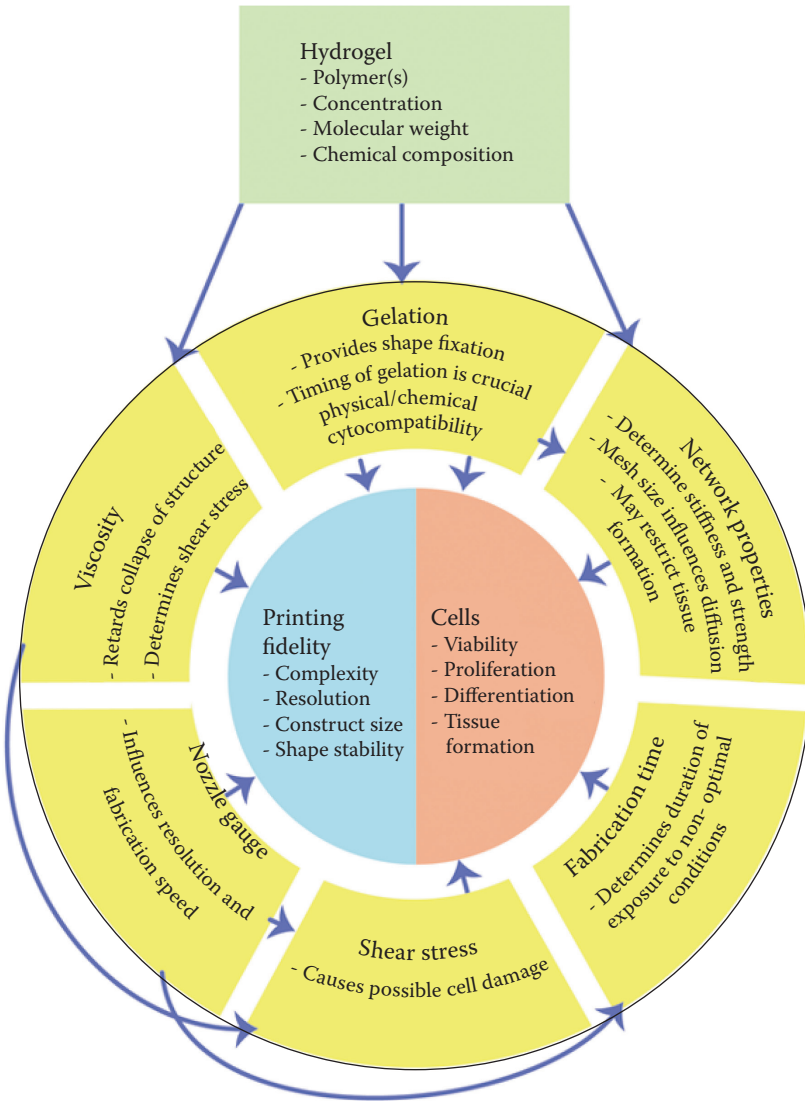


Figure 2.2 Map of concepts and considerations involved in bioprinting. The physical and biochemical properties of the hydrogel, the specific type of cell, and the 3D-bioprinter parameters all factor into the goal of fabricating spatially defined and functional tissues. (From Malda, J. et al.: 25th Anniversary Article: Engineering Hydrogels for Biofabrication. *Advanced Materials*. 2013. 25. 5011–5028. Copyright John Wiley & Sons. Reprinted with permission.)

the tissue of interest. Tissue specificity may be achieved by using tissue-specific decellularized extracellular matrix (dECM) or by functionalizing a nonspecific ink with tissue-specific growth factors. Second, for implanted scaffolds, it is desirable to match the degradation rate of the 3D-printed implant to the rate of tissue regeneration. Mismatched degradation rate may lead to fibrosis or chronic inflammation. Finally, while bioinks themselves must not be cytotoxic, it is also critical to ensure that their degradation by-products are also cytocompatible.

2.5.1 Bioink biocompatibility

First and foremost, candidate bioinks must be biocompatible. Specifically, bioinks must support cell viability, growth, and function. Therefore, it is important to use mild processing conditions that are nontoxic and do not injure cells. Some cross-linking strategies, such as those using harsh chemicals or toxic photoinitiators, may therefore not be ideal for bioinks. Biocompatibility can be assessed by measuring cell viability, for example, with live/dead staining, and proliferation, for example, with the Picogreen or MTS assays.

2.5.2 Bioink printability

Printability with regard to extrusion-based platforms has begun to acquire progressively more quantitative definitions as the field progresses. [Figure 2.3](#) shows the qualitative definitions of printability. A printable biomaterial extrudes in uniform, linear strands from a nozzle, maintains its shape after extrusion, can span gaps, and is self-supporting to enable multilayer printing (Jakus et al. 2015). Aspects that are typically varied in the search for a printable bioink formulation include but are not limited to polymer fraction, cross-link density, cross-linking mechanism, and shear response. As stated previously, a higher polymer fraction can lead to better printability but decreased cell viability. The decreased cell viability can be related to several factors, such as decreased nutrient/waste diffusion and excessive shear forces imparted on the cells. Varying the degree of cross-linking within a hydrogel increases the storage modulus (solid-like properties) without increasing the polymer fraction, but can affect the cell viability and shear stress on extrusion. The specific cross-link mechanism used can also influence printability. Chemically cross-linked gels are less shear-responsive compared to thermally cross-linked inks. Thermally cross-linked bioinks are printed using temperature-controlled 3D printers at temperatures ranging from refrigeration temperatures to body temperatures, to maintain cell viability. Other sophisticated physically cross-linked methods may offer designed shear-thinning properties that liquefy at the nozzle wall but gel immediately after extrusion (Dubbin et al. 2016; Ouyang et al. 2016). Often, bioink formulations may promote high cell viability but are either too weak to support themselves, dissolve in long-term cell culture, or are unable to be

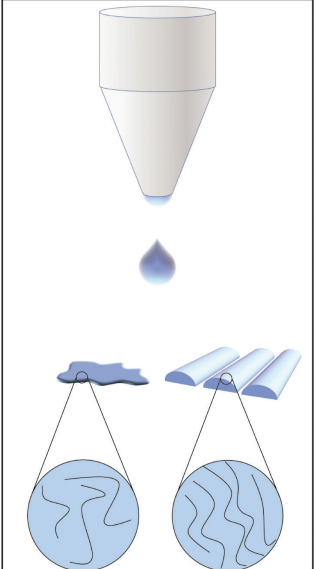
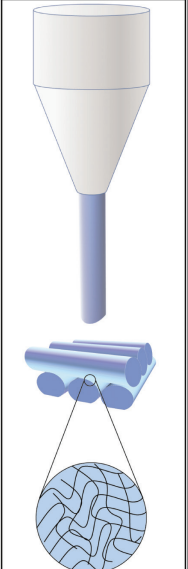
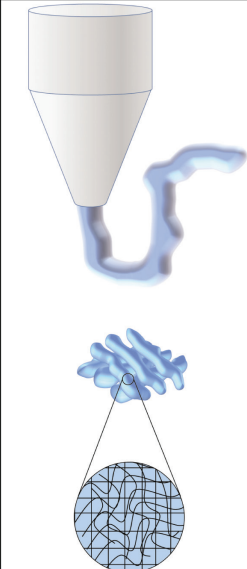
Liquid phase	Ideal	Robust gel
		
• Dropwise extrusion • No self-support • Cell settling • High cell viability • Loose, flowable polymer network	• Laminar extrusion • Self-supporting • Defined geometry • Tolerable shear • Lightly cross-linked polymer network	• Clumpy extrusion • Self-supporting • Undefined geometry • High shear • Densely cross-linked polymer network

Figure 2.3 Varying definitions of printability. Ideal printed inks can extrude in laminar filaments, can span gaps, and can support multiple layers. Nonideal inks are either too soft or too robust, lacking shape fidelity or imparting too much shear stress on encapsulated cells, respectively.

handled. In these cases, scaffolds may be exposed to a secondary cross-linking mechanism after printing to overcome these limitations.

2.5.3 Printer and nozzle properties

Cell viability can be decreased by any number of aspects of a 3D-printing system. Stresses imparted on cells during the printing process often mirror those of flow cytometry or fluorescence-activated cell sorting (FACS). Properties such as mass flow rate, extrusion pressure, nozzle diameter, and nozzle shape can have drastic effects on cell viability. Large diameter ($\sim 400 \mu\text{m}$), conical nozzles have been demonstrated to lead to increased cell viability on extrusion (Figure 2.4). Studies of injectable cell therapies can provide insight into the effects of extrusion parameters on cell viability. The laboratory of Sarah Heilshorn has developed techniques for

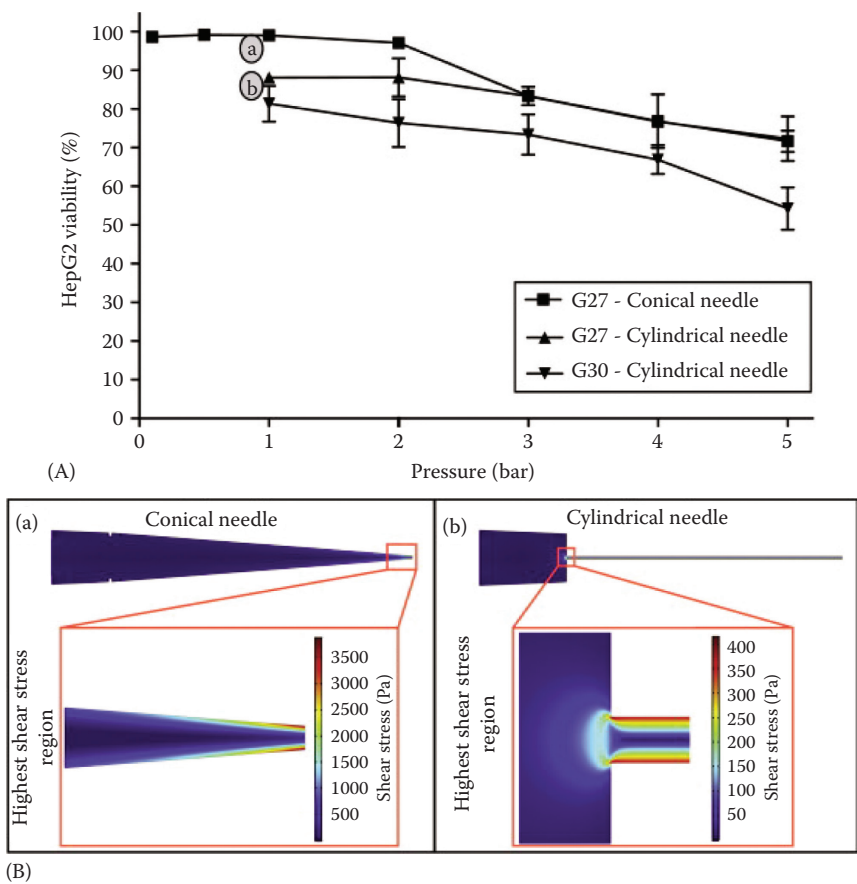


Figure 2.4 (A) cell viability response to needle extrusion at different pressures and nozzle geometries. (B) (a) Finite element model showing heat map of shear stresses at 1 bar of inlet pressure in conical and (b) cylindrical nozzles of 10% (w/v) gelatin. Nozzle inner diameters are 200 μm . (Reprinted from *Biomaterials*, 35, Billiet, T. et al., The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability, 49–62, Copyright 2014, with permission from Elsevier.)

injectable therapies and adapted them into the realm of 3D printing, specifically mixing-induced two component hydrogels (MITCH) (Foo et al. 2009; Cai et al. 2016; Dubbin et al. 2016). In these two-component systems, as opposed to single component, Dubbin et al. observed an increase in cell viability attributed to the addition of shear thinning qualities to the bioink. Injectable cell therapies developed by the laboratory of Jason Burdick have also been adapted to bioprinting (Ouyang et al. 2016; Rodell et al. 2016). The Burdick lab's approach to shear-thinning hydrogels is based on a reversible, self-healing guest–host physical interaction between

adamantane and beta-cyclodextrin. The physical extrusion of a cell-laden bioink through a bioprinter nozzle is arguably the harshest physical insult cells that will be exposed to during the bioprinting process, and reducing this stress (with a wider nozzle, lower pressure, etc.) is a principle concern in bioprinting.

2.6 Natural bioinks

Natural bioinks, or bioinks fabricated from naturally derived polymers, have been widely explored for TE applications. Compared to synthetic bioinks, natural bioinks inherently contain bioactive motifs for cell adhesion, signaling, and degradation. In many cases, natural bioinks have mechanical properties similar to native ECM. However, there are also some disadvantages to natural bioinks, largely related to how the natural polymers are produced. In particular, because they are isolated from natural sources there can be batch-to-batch variation, which may lead to variable printability of bioink formulations. In addition to batch-to-batch variation, naturally derived polymers may provoke an immune response when implanted *in vivo* due to xenogeneic polymer source or cause infection from zoonotic pathogens. Finally, there is often limited tunability of mechanical or degradation properties within natural polymer systems. Often, bioinks are actually mixtures of natural polymers. In this section, we will review some of the commonly used natural polymers for bioprinting and cover topics of polymer structure and properties as well as bioink synthesis, fabrication, and applications.

2.6.1 Protein-based bioinks

Many natural bioinks are made of proteins. To improve mechanical strength or control degradation, protein-based bioinks are often chemically cross-linked. Due to their amino acid building blocks, the same chemical cross-linking strategies can be applied to nearly all proteins and often rely on the two most abundant functional groups in proteins: amines and carboxylic acids. The most common chemical cross-linking reactions for proteins rely on aldehydes or amine–carboxylic acid carbodiimide coupling, both of which are cytotoxic and can therefore not be used for cell-encapsulating bioinks. PEG cross-linkers (bi- or multifunctional) with aldehydes or activated esters have been used for amine–carboxylic acid-based cross-linking in proteins (Figure 2.5) (Rutz et al. 2015), although it must be noted that the PEG remains in the final construct and may affect the final properties. The use of amine-based cross-linking in cell-encapsulating bioinks may also cross-link amines on the surface of the cells to the polymer network and could lead to decreased cell viability due to the potentially high deformation cells may experience during extrusion, especially if linked directly to the polymer network. To utilize other cross-linking reactions, proteins can be functionalized to present a larger variety of functional groups that permit more cell-friendly cross-linking

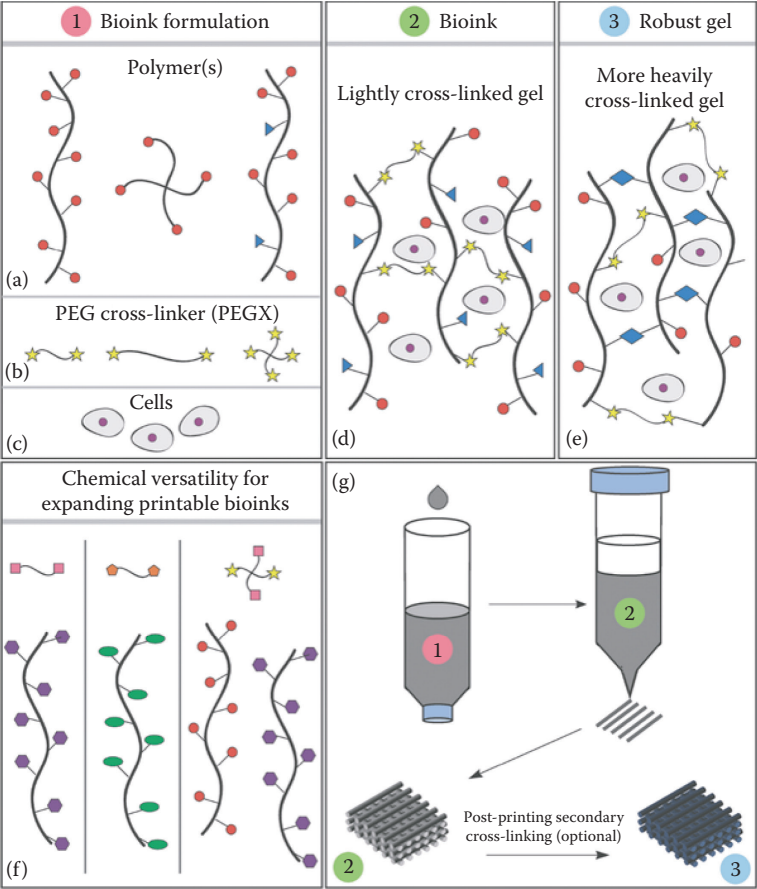


Figure 2.5 Scheme of 3D-printing process described by Rutz et al. (2015). (1) Main components of a bioink include (a) a base polymer(s), (b) a PEG cross-linker (PEGX), and (c) cells, which are lightly cross-linked (d) and extruded into a structure (2, g). An optional secondary cross-linking treatment can stabilize the structure for long-term culture (3, e). For further versatility, the base polymer can be functionalized to present additional reactive groups (f). (From Rutz, A.L. et al.: A Multimaterial Bioink Method for 3D Printing Tunable, Cell-Compatible Hydrogels. *Advanced Materials*. 2015. 27. 1607–1614. Copyright John Wiley & Sons. Reprinted with permission.)

chemistries. For example, amines and carboxylic acids can be converted into the desired functional group by reaction with a bifunctional small molecule. Acrylation or methacrylation has been performed to create UV cross-linkable bioinks (Billiet et al. 2014; Kolesky et al. 2014). Similarly, thiolation can be performed, which enables thiol-ene, thiol-maleimide, and thiol-vinyl sulfone cross-linking reactions, which are considered cell-friendly (Fu et al. 2012; Phelps et al. 2012). Thiol-based

reactions are included in a class of reactions called *click chemistry* (Nimmo and Shoichet, 2011). In the past few years, more cell-friendly click reactions (metal-free, mild-reaction conditions) have been developed and could also be used for bioink synthesis. Most important, functionalization is not only useful to expand potential cross-linking reactions, but it can also be used to incorporate bioactive motifs, drugs, or imaging moieties into bioinks (Zheng et al. 2015).

2.6.1.1 Collagen Collagen is the most abundant protein in the mammalian ECM, and hence is one of the most widely used natural polymers in TE. Collagen has cell adhesion (RGD) sites and can be degraded by cellular matrix metalloproteases. The collagen molecule consists of three polypeptide chains that wrap around one another to form a triple helix, held together by covalent bonds. Collagen molecules self-assemble into collagen fibrils. Collagen can be solubilized by partial digestion with acid and/or enzymes. When this solubilized collagen solution is neutralized and heated (20°C–37°C), collagen monomers and oligomers self-assemble into a physically cross-linked hydrogel. As collagen forms physically cross-linked gels at physiological conditions, collagen can be used as-is for (cell-encapsulating) bioink synthesis. Atelocollagen, a water-soluble form of collagen generated by protease treatment, retains virtually all the properties of collagen. Unfortunately, a severe limitation of collagen and atelocollagen bioinks is their poor mechanical strength. High weight fraction collagen inks may display enhanced mechanical properties but are not suitable for cell encapsulation because dense polymer networks impede nutrient/waste diffusion and restrict cell spreading and migration. Collagen hydrogels can be made more robust through treatment with chemical cross-linkers, such as carbodiimide and glutaraldehyde, but as mentioned previously these chemical cross-linkers can be cytotoxic. In spite of these undesirable mechanical properties, collagen has been successfully 3D printed, using various support strategies. More cell-friendly chemical cross-linkers, such as tannic acid (Yeo and Kim 2017), genipin (Kim et al. 2016), or vitamin B2 (Diamantides et al. 2017), have been used to simultaneously achieve adequate shape fidelity and good cell viability. Hinton et al. (2015) demonstrated high cell viability when printing collagen and collagen-containing multicomponent bioinks into a gelatin microparticle support bath. During printing, the gelatin serves as a temporary, thermoreversible support to maintain the intended structure during printing; after printing, the gelatin support can be washed away by warming to 37°C. Similarly, Lee et al. (2010) coprinted a collagen hydrogel precursor bioink, which was cross-linked with aerosolized sodium bicarbonate, alongside a sacrificial gelatin ink. Shim et al. (2011) and Lee et al. (2014b) have demonstrated, in numerous examples, the high viability of cell-laden solution-phase bioinks, including an atelocollagen bioink. These inks undergo a slow thermal physical cross-linking and therefore require coprinting a hot-melt PCL–PLGA support structure to achieve shape fidelity of the bioinks. Yeo et al. (2016) showed high cell viability within a collagen bioink by coprinting with alginate using a coaxial nozzle. Each printed filament consists of a collagen

core and a surrounding alginate sheath, which is cross-linked immediately after extrusion in a calcium chloride mist.

2.6.1.2 Gelatin Gelatin is derived through partial hydrolysis of collagen. Gelatin retains many features of collagen including cell adhesion (RGD) sites, cell degradation (MMP-cleavable) sites, and the ability to form triple helices. However, it is unable to form long fibrils. Instead, local regions of triple helices on different gelatin strands interact to form physical cross-links that are responsible for gelation. Gelation of gelatin is thermoreversible; it is a gel at cold temperatures and a solution at physiologic temperature. Gelatin can be easily dissolved in warm water or buffer without the need for acids or enzymes. In summary, gelatin retains many of the bioactivity of collagen, but it is simpler to use.

As gelatin is a solution at physiologic temperature, chemical cross-linking is necessary for cell culture and *in vivo* studies. Carbodiimide chemistry has also been used to cross-link 3D-printed gelatin scaffolds, but as previously mentioned, it would be inappropriate for bioinks due to its cytotoxicity. Wang et al. (2006) took advantage of gelatin's thermal physical cross-linking, by printing hepatocyte-laden gel-phase gelatin inks at room temperature. Post-printing cross-linking was conducted using glutaraldehyde, and while they reported viability >90% after up to one month of culture, concerns remain about the cytotoxicity with glutaraldehyde. More recently, Rutz et al. (2015) demonstrated a cell-friendly cross-linking reaction using homobifunctional PEG-succinimidyl valerate (SVA) to cross-link the naturally occurring amines in gelatin. Gelatin/PEG-SVA bioinks were printed as lightly cross-linked gels, which prevented cell settling and maintained high cell viability. Gelatin can easily be functionalized to expand cross-linking options beyond amines and carboxylic acids. The most commonly used form of functionalized gelatin is gelatin methacrylate (GelMA), which can be photocross-linked. Kolesky et al. (2014) demonstrated good cell viability when coprinting photocross-linked GelMA bioinks and fugitive Pluronic inks (for support). Khademhosseini and colleagues have demonstrated, in numerous examples, the ability to print GelMA either alone or in combination with alginate, using conventional and coaxial nozzles (Jia et al. 2016; Liu et al. 2017a). Billiet et al. (2014) demonstrated the ability to print thermally physically cross-linked gel-phase GelMA bioinks with good cell viability. Liu et al. printed novel alginate-GelMA composite hydrogels containing alginate lyase, an enzyme that degrades alginate (Liu et al. 2017b). During printing, shape fidelity was maintained by the fast physical cross-linking of the alginate and the relatively high polymer fraction of the ink; over time, however, the enzyme-mediated degradation lead to the removal of the alginate matrix, leaving behind only the GelMA and resulting in improved bioactivity of the scaffold as evidenced by enhanced cell spreading and proliferation. Gelatin acrylate bioinks have better reaction kinetics compared to GelMA, are more mechanically robust, and have higher cell viability (Billiet et al. 2013).

Thiolation of gelatin results in bioinks that can be chemically cross-linked in a more cell-friendly, bio-orthogonal manner using acrylates, alkynes, maleimides, or vinyl sulfones. For example, Skardal et al. (2015) used two distinct reactions: thiol–acrylate and UV-activated thiol–alkyne, to create a final construct with tunable stiffness. Thiol–acrylate cross-linking yielded a lightly cross-linked bioink, which could be extruded into self-supporting, pattern-maintaining filaments; UV-activated thiol–alkyne cross-linking was used as a post-printing processing step to tailor stiffness. Allyl-functionalized gelatin (*GelAGE*) has been cross-linked via a thiol-ene click reaction to produce a bioink that supports long-term viability of encapsulated chondrocytes post-printing (Bertlein et al. 2017). Sakai et al. used a mixture of phenol-functionalized gelatin (Gelatin–Ph) and hyaluronic acid (HA–Ph) in the presence of ruthenium(II) tris-bipyridyl dication and sodium ammonium persulfate for visible light-initiated cross-linking (Sakai et al. 2017). Within this bioink, encapsulated human adipose-derived stem cells (hADSCs) had excellent viability (>95% at 24 hours), proliferated in the hydrogel, and differentiated into adipocytes when cultured in adipogenic differentiation medium. Another strategy to maintain 3D-printed gelatin constructs at physiologic conditions is to incorporate another polymer, with more favorable cross-linking, into the gelatin bioink. Ouyang et al. (2015) printed embryonic stem cell (ESC)-encapsulating gelatin–alginate bioinks at room temperature, where the gelatin forms a gel and cross-linked post-printing with calcium chloride. Within these constructs, they observed viability >90%, proliferation, and maintenance of pluripotent phenotype of the ESCs.

2.6.1.3 Fibrin/fibrinogen Fibrin, the polymer that makes up blood clots, is formed through thrombin-mediated cleavage of fibrinogen. Following cleavage, fibrin monomers self-assemble into fibrils, which are stabilized by Factor XIII, transglutaminase, that links glutamine and lysine residues. Fibrin hydrogels can be fabricated, just as in nature, by mixing fibrinogen, thrombin, and calcium. Fibrinogen is easily isolated from blood, meaning that fibrinogen can be isolated from a patient’s own blood for autologous therapy, thereby reducing the risks of foreign body reactions (Buchta et al. 2005). Moreover, there is no added cost to using patient-derived fibrinogen compared to commercially available fibrinogen (Ahmed et al. 2008). Fibrin has an excellent bioactivity, with motifs for cell adhesion, heparin binding, ECM protein binding, and growth factor binding.

Fibrin hydrogels are degraded quickly because they contain motifs for *both* plasmin and matrix metalloprotease degradation. Fibrin degradation can be slowed by incorporation of fibrinolytic inhibitors, such as aprotinin (Le Guehennec et al. 2007) or covalent cross-linking with peptides (Sacchi et al. 2014). Tranquillo et al. demonstrated a cell-friendly blue light photocross-linking reaction to increase fibrin stiffness (Syedain et al. 2009; Bjork et al. 2011). Kang et al. (2016) used a multicomponent hydrogel bioink, composed of gelatin, fibrinogen, hyaluronic

acid, and glycerol, and demonstrated alignment of myoblasts (muscle-precursor cells) along the printed filament. Shape fidelity was enhanced by support materials, including a polycaprolactone (PCL) framework and a sacrificial Pluronic ink. Post-printing cross-linking relied on thrombin-mediated fibrin cross-linking and molecular entanglements to hold together the other polymer components. Kolesky et al. (2016) fabricated gelatin–fibrinogen bioinks and cross-linked them using a dual-enzyme strategy with thrombin and transglutaminase. As with other work by Kolesky et al. (2016), the protein-based bioink is coprinted with a silicone ink and a Pluronic ink for support and to improve shape fidelity.

2.6.2 Polysaccharide-based bioinks

2.6.2.1 Alginate Alginate, a polysaccharide derived from brown seaweed, is a linear copolymer of alpha-L-guluronic acid (G) and (1-4)-linked beta-D-mannuronic acid (M) monomers. The monomers may be arranged in alternating or repeating blocks, depending on the species, age, and environmental conditions of the seaweed (Johnson et al. 1997; Donati et al. 2009). Alginate has low toxicity and gels via gentle physical cross-linking, whereby divalent cations (most commonly calcium) interact with G monomer blocks to form ionic bridges between different polymer chains. As mentioned previously, physical cross-linking is reversible and ionic cross-linking is particularly vulnerable to dissolution in culture or *in vivo* where local divalent cation concentrations may draw cross-linking calcium out of the gel, leading to gradual degradation of alginate gels over time. As it is derived from algae, alginate lacks cell binding or degradation motifs for mammalian cells. Due to this lack of bioactivity, alginate is not typically used alone in bioinks, rather it is added to multicomponent bioinks because it is easily, quickly, and gently cross-linked by the addition of calcium ions. For enhanced bioactivity, RGD peptide-coupled alginate can be used. For example, Cuniffe et al. used a bioink formulation consisting of RGD–alginate, nanohydroxyapatite complexed to plasmid DNA, and bone marrow-derived mesenchymal stem cells printed within a PCL framework for bone TE (Cuniffe et al. 2017). This bioink supported satisfactory cell viability and the addition of plasmid DNA improved biological functionality by enhancing vascularization and mineralization of the printed construct when implanted subcutaneously. Finally, Ahlfeld et al. developed composite bioink made from alginate and Laponite, a synthetic clay known for its favorable drug delivery properties, to encapsulate human mesenchymal stem cells for bone TE applications (Ahlfeld et al. 2017). The addition of the Laponite not only permitted controlled delivery of bovine serum albumin (BSA) and vascular endothelial growth factor (VEGF) but also improved shape fidelity of the printed construct. Most important, good cell viability was maintained over 21 days.

2.6.2.2 Hyaluronic acid Hyaluronic acid (HA) is a linear polysaccharide composed of a repeating disaccharide of beta-D-glucuronic acid and *N*-acetyl-beta-D-glucosamine monomers. HA is found in almost every mammalian tissue and fluid. HA hydrogels can be covalently cross-linked with hydrazide derivative, polyfunctional epoxides, carbodiimides, self-assembly, and esterification (Collins and Birkinshaw 2013). HA contains hydroxyl and acetamide functional groups, which can be used for cross-linking or modified to permit alternative cross-linking chemistries or bioactive molecule tethering (Khan and Ahmad 2013). Photocross-linkable methacrylated HA has been incorporated as a stabilizing agent in gelatin and other multicomponent inks (Kesti et al. 2015). Ouyang et al. (2016) developed a shear-thinning, self-healing bioink using guest–host bonding. By coupling adamantane (guest) or beta-cyclodextrin (host) moieties to separate batches of HA, the two HA batches gel by supramolecular assembly on mixing. The mechanical strength of the 3D-printed construct was enhanced by incorporating methacrylated HA and performing post-printing UV-cross-linking (2016). HA is highly hydrated and is, therefore, often added to bioink formulations to increase viscosity for improved printability. For example, Kang et al. (2016) used multicomponent bioink, composed of gelatin, fibrinogen, HA, and glycerol for skeletal muscle reconstruction. HA and glycerol were added to the bioink formulation to improve printability (better uniformity and less nozzle clogging) and to shield cells from the shear stress imposed by the printing process. Even with the increased viscosity due to the presence of HA, Kang et al. (2016) printed the multicomponent bioink using an internal hot-melt PCL framework and external Pluronic sacrificial scaffold to improve the shape fidelity of the printed hydrogel bioink. After printing, the fibrinogen in the bioink was cross-linked with thrombin and uncross-linked components (gelatin, HA, glycerol, and the Pluronic support ink) were washed away. Thiol-functionalized hyaluronic acid (HA–SH) has been cross-linked with allyl-functionalized poly(glycidol)s (P(AGE-co-G)) via UV-induced radical thiol-ene coupling (Stichler et al. 2017). By adding additional unfunctionalized HA, Stichler et al. increased the viscosity of the bioink without diminishing the viability of the encapsulated human or equine chondrocytes. However, even with this additional viscosity, a PCL framework was needed to achieve desired shape fidelity with this HA–SH/P(AGE-co-G) bioink.

2.6.2.3 Chitosan and chitin Chitosan and chitin are related shellfish-derived polysaccharide copolymers, composed of randomly or block-distributed *N*-acetylglucosamine and *N*-glucosamine monomers (Khor and Lim 2003). Chitin is insoluble in most common solvents, and therefore, it is usually converted to chitosan by deacetylation, making it more soluble. Chitosan can be degraded by mammalian enzymes (e.g., lysozyme) and is structurally similar to mammalian glycosaminoglycans. Chitosan can be dissolved in dilute acids and gels at neutral pH. Gu et al. (2016) utilized a multicomponent bioink composed of alginate, carboxymethyl–chitosan,

and agarose for bioprinting human neural stem cells. Most important, the ink maintained a homogenous distribution of cells throughout the printing process. Agarose increased the viscosity of the bioink, making it suitable for 3D printing. The alginate component was critical for the post-printing ionic cross-linking, and chitosan-promoted cell viability. The ratio of alginate:agarose:chitosan could be manipulated to tune the mechanical properties of the bioink, to study how stiffness affects the neural stem cell behavior (Gu et al. 2016).

2.6.3 Other natural bioinks

2.6.3.1 Matrigel™ Matrigel has garnered a unique reputation within the TE field. Its bioactivity in capillary formation assays and use in stem cell biology research has emphasized the necessary role of ECM in differentiation (Arnaoutova et al. 2009). However, its source (EHS mouse tumor) precludes any possibility of translatability. Nevertheless, it holds potential to create *in vitro* tissues for development and disease modeling research. 3D-printing Matrigel has proved challenging; even in its fully gelled state it displays less than an ideal printability using extrusion-based methods. This was elegantly demonstrated by Poldervaart et al. (2014) whereby increasing bioactivity (Matrigel weight percent) yielded decreased printability, whereas increasing printability (Alignate weight percent) yielded decreased bioactivity and cell viability. [Figure 2.6](#) succinctly demonstrates the inverse relationship between printability and cell compatibility. Cells encapsulated within Matrigel were extruded at pregel temperatures (0°C–4°C) into a microfluidic polydimethylsiloxane (PDMS) chamber (Snyder et al. 2011). Although cell function and viability were high, this approach did not achieve multiple layers. Patterning of Matrigel has received some success using laser-induced forward transfer (LIFT) and laser-guided direct writing; however, these have limitations for fabricating large, 3D constructs and are outside the scope of this chapter (Nahmias et al. 2005).

2.6.3.2 Decellularized extracellular matrix hydrogels dECM hydrogels have received an enormous amount of attention from the TE community in recent years (Saldin et al. 2016). They have the potential to be free from immunogenic cellular material while retaining natural protein matrix enabling cellular attachment in addition to certain matrix-bound signaling factors. The specific components of a dECM batch and its resulting hydrogel have been known to be extremely variable. The specific decellularization protocol in question typically involves harsh treatments with detergents and hypertonic salt solutions, which can lead to modifications in residual matrix content, removal of signaling factors, and deformation of protein's tertiary and quaternary structures (Keane et al. 2012). The residual protein content within dECM hydrogels is an active area of investigation of several labs. While the batch-to-batch variability is a known drawback, the organ specificity of dECM hydrogels is one of the major advantages in their application. Liver dECM may contain a specific

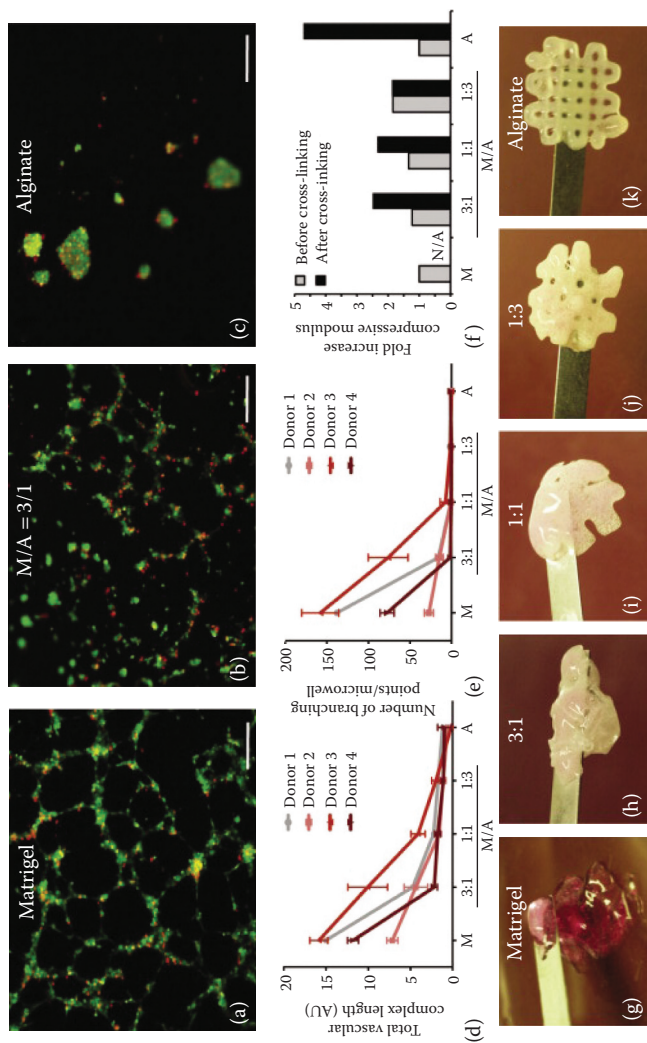


Figure 2.6 Demonstration of the balancing act between printability and bioactivity. (a–c) Increasing Matrigel content has a positive relationship with viability and tubule formation of cultured endothelial cells. Matrigel is a natural polymer, so it exhibits some batch-to-batch variability, demonstrated here by comparing the (d) vascular length and (e) number of branch points. (f) The composition of the Matrigel-alginate ink affects its compressive modulus. (g–k) Increasing alginate content has a positive relationship with printability and shape fidelity. Obtaining a functional 3D-printed structure requires compromise between shape fidelity and cell viability/function. (Reprinted from *J. Control. Release*, 184, Poldervaart, M.T. et al., Prolonged presence of VEGF promotes vascularization in 3D bioprinted scaffolds with defined architecture, 58–66. Copyright 2014, with permission from Elsevier.)

combination of matrix proteins and signaling factors that can increase hepatocyte function and/or stem cell differentiation compared to a decellularized kidney, for example (Nakayama et al. 2010; Lee et al. 2014a). These factors have implications in downstream processing when the solution is solubilized, typically by enzymatic digestion at a reduced pH. The modification in bioactivity postdigestion has not been rigorously investigated; enzymatic digestion is part of proteomics processing and the presence of a peptide sequence does not necessarily imply bioactivity. Similarly, ELISA or Western analysis also recognizes specific peptide sequences; however, they have the potential to better assess the protein structure if the recognized epitope is conformation dependent. The variability in structural protein content due to processing (e.g., decellularization or digestion method) and organ source (species, age, organ, and disease) can all influence the resulting hydrogel's mechanical properties and thus printability, in addition to the hydrogel's bioactivity.

While the exact contents of dECM hydrogels are elusive for the time being, they have nevertheless demonstrated significant results. After the dECM is digested, the resulting digest is normally kept at low ($\sim 4^{\circ}\text{C}$) temperatures until use. Similar to collagen hydrogels, when dECM digest is pH-neutralized, it will begin to gel at room temperature when collagen fibrils begin to reassemble; however, this process is slightly accelerated at 37°C (Freytes et al. 2008; Lonekera et al. 2015; Saldin et al. 2016). These gelation kinetics are not ideal for 3D-printing applications, necessitating workaround solutions that have applications in other liquid-phase bioink printing systems. Cho et al. have demonstrated numerous permutations of a method to create 3D structures using dECM hydrogels by coprinting a hot-melt thermoplastic, poly (caprolactone) (PCL), for applications in breast (Pati et al. 2015), heart (Pati et al. 2014) (Jang et al. 2017), osteochondral (Jang et al. 2017), and liver (Cho et al. 2016; Lee et al. 2017) TE. A PCL structure is first 3D printed into a single layer, which is then followed by extrusion of the cell-laden dECM pregel in between the PCL struts. The dECM is then allowed to gel at 37°C . Organ and tissue-specific cellular function has been demonstrated using this approach; however, this is likely a property of the bioactive dECM components and not of the printing itself. An exception is the extrusion of muscle-derived dECM, which has demonstrated alignment of myofibrils dependent on extrusion nozzle size (Choi et al. 2016). Kang et al. (2016) were able to demonstrate a similar myotube alignment phenomenon also using a PCL support structure. Drawbacks of using a thermoplastic support structure to obtain a 3D-printed structure include impaired biodegradation and, most notably, mechanical property mismatch of the target tissues. Jang and colleagues (2016) did however manage to 3D-print dECM hydrogels laden with high concentrations of vitamin B2, which later underwent UVA exposure for secondary cross-linking. Skardal et al. (2015) also managed to use a complex mixture of modified gelatin, hyaluronic acid, and polyethylene glycol, along with UV exposure, to print dECM hydrogels. In addition, Jang et al. (2016) demonstrated UVA-induced cross-linking of vitamin B2-rich scaffolds as a secondary cross-linking method. This method was

used to print liquid-phase dECM hydrogels, but demonstrated decreased cell viability with an increasing B2 content. Athitasala et al. demonstrated that composite hydrogels composed of alginate and dentin-derived hydrogel supported good viability of odontoblast-like cells (OD21s) for at least 5 days post-printing (Athirasala et al. 2017). Moreover, they optimized the alginate:dentin ratio to yield the best print accuracy and cell differentiation (highest expression of ALP and RUNX2). None of the preceding methods, however, demonstrated large, self-supporting structures with the hydrogel ink alone. The difficulty with which dECM hydrogels are bio-printed has led to the above work-around solutions. Development of self-supporting dECM bioinks that retain native bioactivity is likely to be a daunting task that will nevertheless be a breakthrough once it is achieved.

2.6.3.3 Scaffold-free bioprinting The printability requirements of bioprinting often necessitate that a bioink be comprised mostly of a hydrogel biomaterial. However, the relatively ECM-sparse cell-dense nature of some tissues led researchers to explore 3D printing of dense cellular aggregates (Norotte et al. 2009). Many metabolically active cells tend to form a necrotic core in aggregation due to limited nutrient diffusion (Lin and Chang 2008). Utilization of relatively quiescent primary isolated cells, or terminally differentiated cells, can help alleviate this problem. Once adequate cell numbers are procured, cells are concentrated and shaped into spheroids using well plates (Tan et al. 2014), by uniformly cutting an aggregated strand of cells (Jakab et al. 2008), or by mechanical disruption of cell sheets (Bakirci et al. 2017). Aggregates can then be deposited using a bioprinter within a supportive gel matrix, enabling the aggregates to fuse to a specific shape. Heterogeneous mixtures of cells, such as hepatocytes and endothelial cells, can encourage capillary formation inside aggregates. This method has been established by the laboratory of Gabor Forgacs and has since then found several industrial applications in *in vitro* tissue modeling (Organovo™) and engineered leather production (Modern Meadow™) (Khatiwala et al. 2012; Gruner and Tyrrell 2015). The scaffold-free approach, however, has limitations in creating organs that require vasculature larger than capillaries (venules and arterioles), and the spatial resolution of this approach is limited by the size of the aggregates. In addition, human-scale organ engineering using this approach will require a massive starting amount of cells, whereas other strategies encourage tissue formation and remodeling starting from a relatively small cell number with the intention of the cells proliferating *in vitro* or after *in vivo* implantation.

2.7 Synthetic bioinks

Polyethylene glycol (PEG) has become a heavily used biomaterial in TE (Zhu 2010). PEG is relatively easily chemically modifiable, allowing it to be tailored with specific physical and chemical properties that are suitable for

distinct applications. However, PEG itself lacks cell attachment sites, its hydrophilic nature prevents protein adhesion, and it is not inherently biodegradable. These drawbacks have led researchers to incorporate specific degradation sites (chemical or enzymatic) as well as specific bioactive peptides tailored to the application (Zhu 2010). Of note is that the molecular weights of the degradable PEG components must be small enough for renal clearance if the system is to be implanted *in vivo*. RGD-doped PEGDA demonstrated improved viability over PEGDA when patterned within a lobule-like structure (Liu Tsang et al. 2007). In extrusion-based systems, modified PEG has been used as a tunable primary cross-linking mechanism to form cell-encapsulating gels prior to extrusion (Rutz et al. 2015; Skardal et al. 2015). Both methods utilized secondary cross-linking mechanisms, such as UV exposure, for long-term culture. However, the method developed by Rutz et al. (2015) demonstrated multilayer self-supporting structures and higher spatial resolution on printing. Skardal et al. (2010) also demonstrated synthesis and acrylation of a four-arm PEG cross-linker capable of rapid gelation and high cell viability on extrusion. This method required agarose support structures, however. In another example, inks composed solely of PEGDA were 3D printed alongside a PEGDA/alginate mixture under continuous UV exposure to obtain mechanically heterogeneous constructs for heart valve TE (Hockaday et al. 2012). Hockaday et al. also tailored the mechanical properties by using different molecular weight PEGDA. The lack of adhesion sites on PEGDA, however, necessitated inclusion of solubilized collagen I. Laponite (synthetic clay) nanoparticles have also been added to PEG bioinks to increase viscosity, storage modulus, and network stability (Peak et al. 2017). Most important, PEG–Laponite hydrogels have shear-thinning and self-healing properties, making them excellent gel-phase bioinks. Lorson et al. reported a series of thermogelling synthetic block copolymers composed of hydrophilic poly(2-methyl-2-oxazoline) (PMeOx) and the thermo-responsive poly(2-n-propyl-2-oxazoline) (PnPrOx) (Lorson et al. 2017). These block copolymers are excellent candidate bioinks due to their cell-friendly cross-linking conditions, high strength ($G' > 1000$ Pa), transparency, and shear thinning properties. Moreover, they support high viability of fibroblasts post-printing. However, lack of bioactivity, in the form of cell adhesion domains, remains disadvantage of synthetic polymers.

2.8 Conclusion

Significant advances within the field of bioink fabrication and 3D printing have been made within the past several years. The number of bioink formulations available has expanded tremendously, addressing the relatively sparse palette of materials for 3D bioprinting that were initially available. Researchers have thus begun to take advantage of the multitude of inks in development by fabricating complex

tissues using these new technologies. Recreating organs for transplant using 3D bioprinting necessitates precise placement of specific cells, materials, and bioactive factors to induce functional tissue formation and regeneration. Attempting to meet these requirements for more complex tissues emphasizes the need for more tissue-specific, biologically and mechanically tunable bioinks. As the number of bioinks continues to grow, so will the knowledge of tissue regeneration and morphogenesis, leading inevitably to clinical translation of new therapies and engineered functional tissues and organs.

References

- Ahlfeld, T., G. Cidonio, D. Kilian, S. Duin, A.R. Akkineni, J.I. Dawson, S. Yang, A. Lode, R.O.C. Oreffo, and M. Gelinsky. 2017. Development of a clay based bioink for 3D cell printing for skeletal application. *Biofabrication* 9 (3): 34103. doi:10.1088/1758-5090/aa7e96.
- Ahmed, T.A.E., E.V. Dare, and M. Hincke. 2008. Fibrin: A versatile scaffold for tissue engineering applications. *Tissue Engineering Part B: Reviews* 14 (2): 199–215. doi:10.1089/ten.teb.2007.0435.
- Arnaoutova, I., J. George, H.K. Kleinman, and G. Benton. 2009. The endothelial cell tube formation assay on basement membrane turns 20: State of the science and the art. *Angiogenesis* 12: 267–274. doi:10.1007/s10456-009-9146-4.
- Athirasala, A., A. Tahayeri, G. Thrivikraman, C.M. Franca, N. Monteiro, V. Tran, J. Ferracane, and L. Bertassoni. 2017. A dentin-derived hydrogel bioink for 3D printing of cell laden scaffolds for regenerative dentistry. *Biofabrication*, November. doi:10.1088/1758-5090/aa9b4e.
- Bakirci, E., B. Toprakhisar, M.C. Zeybek, G.O. Ince, and B. Koc. 2017. Cell sheet based bioink for 3D bioprinting applications. *Biofabrication* 9 (2). IOP Publishing: 24105. doi:10.1088/1758-5090/aa764f.
- Bertlein, S., G. Brown, K.S. Lim, T. Jungst, T. Boeck, T. Blunk, J. Tessmar, G.J. Hooper, T.B.F. Woodfield, and J. Groll. 2017. Thiol-ene clickable gelatin: A platform bioink for multiple 3D biofabrication technologies. *Advanced Materials* 29 (44): 1703404. doi:10.1002/adma.201703404.
- Billiet, T., B.V. Gasse, E. Gevaert, M. Cornelissen, J.C. Martins, and P. Dubruel. 2013. Quantitative contrasts in the photopolymerization of acrylamide and methacrylamide-functionalized gelatin hydrogel building blocks. *Macromolecular Bioscience* 13 (11): 1531–1545. doi:10.1002/mabi.201300143.
- Billiet, T., E. Gevaert, T. De Schryver, M. Cornelissen, and P. Dubruel. 2014. The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. *Biomaterials* 35 (1): 49–62. doi:10.1016/j.biomaterials.2013.09.078.
- Bjork, J.W., S.L. Johnson, and R.T. Tranquillo. 2011. Ruthenium-catalyzed photo cross-linking of fibrin-based engineered tissue. *Biomaterials* 32 (10): 2479–2488. doi:10.1016/j.biomaterials.2010.12.010.
- Buchta, C., H.C. Hedrich, M. Macher, P. Höcker, and H. Redl. 2005. Biochemical characterization of autologous fibrin sealants produced by CryoSeal® and Vivostat® in comparison to the homologous fibrin sealant product Tissucol/Tisseel®. *Biomaterials* 26 (31): 6233–6241. doi:10.1016/j.biomaterials.2005.04.014.
- Cai, L., R. E. Dewi, A.B. Goldstone, J.E. Cohen, A.N. Steele, Y.J. Woo, and S.C. Heilshorn. 2016. Regulating stem cell secretome using injectable hydrogels with in situ network formation. *Advanced Healthcare Materials* 5 (21): 2758–2764. doi:10.1002/adhm.201600497.

- Cho, J.W.L., Y.J. Choi, W.J. Yong, F. Pati, J.H. Shim, K.S. Kang, I.H. Kang, J. Park, and D.W. Cho. 2016. Development of a 3D cell printed construct considering angiogenesis for liver tissue engineering. *Biofabrication* 8 (1). IOP Publishing: 15007. doi:10.1088/1758-5090/8/1/015007.
- Choi, Y.J., T.G. Kim, J. Jeong, H.G. Yi, J.W. Park, W. Hwang, and D.W. Cho. 2016. 3D cell printing of functional skeletal muscle constructs using skeletal muscle-derived bioink. *Advanced Healthcare Materials* 1–10. doi:10.1002/adhm.201600483.
- Collins, M.N., and C. Birkinshaw. 2013. Hyaluronic acid based scaffolds for tissue engineering—A review. *Carbohydrate Polymers* 92 (2): 1262–1279. doi:10.1016/j.carbpol.2012.10.028.
- Cunniffe, G.M., T. Gonzalez-Fernandez, A. Daly, B.N. Sathy, O. Jeon, E. Alsberg, and D.J. Kelly. n.d. Three-dimensional bioprinting of polycaprolactone reinforced gene activated bioinks for bone tissue engineering. *Tissue Engineering Part A*. doi:10.1089/ten.tea.2016.0498.
- Diamantides, N., L. Wang, T. Pruiksma, J. Siemiatkoski, C. Dugopolski, S. Shortkroff, S. Kennedy, and L.J. Bonassar. 2017. Correlating rheological properties and printability of collagen bioinks: The effects of riboflavin photocrosslinking and pH. *Biofabrication* 9 (3): 34102. doi:10.1088/1758-5090/aa780f.
- Donati, I., Y.A. Mørch, B.L. Strand, G. Skjåk-Bræk, and S. Paoletti. 2009. Effect of elongation of alternating sequences on swelling behavior and large deformation properties of natural alginate gels. *The Journal of Physical Chemistry B* 113 (39): 12916–12922. doi:10.1021/jp905488u.
- Dubbin, K., Y. Hori, K.K. Lewis, and S.C. Heilshorn. 2016. Dual-stage crosslinking of a gel-phase bioink improves cell viability and homogeneity for 3D bioprinting. *Advanced Healthcare Materials* 5 (19): 2488–2492. doi:10.1002/adhm.201600636.
- Ferris, C.J., K.J. Gilmore, S. Beirne, D. McCallum, G.G. Wallace, and Marc in het Panhuis. 2013. Bio-ink for on-demand printing of living cells. *Biomaterials Science* 1 (2): 224. doi:10.1039/c2bm00114d.
- Foo, W.P.C.T.S., J.S. Lee, W. Mulyasmita, A. Parisi-Amon, and S.C. Heilshorn. 2009. Two-component protein-engineered physical hydrogels for cell encapsulation. *Proceedings of the National Academy of Sciences* 106 (52): 22067–22072. doi:10.1073/pnas.0904851106.
- Freytes, D.O., J. Martin, S.S. Velankar, A.S. Lee, and S.F. Badylak. 2008. Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. *Biomaterials* 29 (11): 1630–1637. doi:10.1016/j.biomaterials.2007.12.014.
- Fu, Y., K. Xu, X. Zheng, A.J. Giacomini, A.W. Mix, and W.J. Kao. 2012. 3D cell entrapment in crosslinked thiolated gelatin-poly(ethylene glycol) diacrylate hydrogels. *Biomaterials* 33 (1): 48–58. doi:10.1016/j.biomaterials.2011.09.031.
- Gruner, G., and J. Tyrrell. 2015. An interview with Gabor Forgacs: From theoretical physics to the business of 3D bio-printing. *Translational Materials Research* 2 (4). doi:Artn040203/r10.1088/2053-1613/2/4/040203.
- Gu, Q., E. Tomaskovic-Crook, R. Lozano, Y. Chen, R.M. Kapsa, Q. Zhou, G.G. Wallace, and J.M. Crook. 2016. Functional 3D neural mini-tissues from printed gel-based bio-ink and human neural stem cells. *Advanced Healthcare Materials* 5 (12): 1429–1438. doi:10.1002/adhm.201600095.
- Hinton, T.J., Q. Jallerat, R.N. Palchesko, J.H. Park, M.S. Grodzicki, H.-J. Shue, M.H. Ramadan, A.R. Hudson, and A.W. Feinberg. 2015. Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances* 1 (9): e1500758–e1500758. doi:10.1126/sciadv.1500758.
- Hockaday, L.A., K.H. Kang, N.W. Colangelo, P.Y.C. Cheung, B. Duan, E. Malone, J. Wu et al. 2012. Rapid 3D printing of anatomically accurate and mechanically heterogeneous aortic valve hydrogel scaffolds. *Biofabrication* 4: 35005. doi:10.1088/1758-5082/4/3/035005.

- Jakab, K., C. Norotte, B. Damon, F. Marga, A. Neagu, C.L. Besch-Williford, A. Kachurin, et al. 2008. Tissue engineering by self-assembly of cells printed into topologically defined structures. *Tissue Engineering Part A* 14 (3): 413–421. doi:10.1089/tea.2007.0173.
- Jakus, A.E., A.L. Rutz, and R.N. Shah. 2015. Advancing the field of 3D biomaterial printing. *Biomedical Materials* 0. IOP Publishing: 14102. doi:10.1088/1748-6041/11/1/014102.
- Jang, J., T.G. Kim, B.S. Kim, S.W. Kim, S.M. Kwon, and D.W. Cho. 2016. Tailoring mechanical properties of decellularized extracellular matrix bioink by vitamin B2-induced photo-crosslinking. *Acta Biomaterialia* 33: 88–95. doi:10.1016/j.actbio.2016.01.013.
- Jang, J., H.J. Park, S.W. Kim, H. Kim, J.Y. Park, S.J. Na, H.J. Kim et al. 2017. 3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair. *Biomaterials* 112: 264–274. doi:10.1016/j.biomaterials.2016.10.026.
- Jia, W., P.S. Gungor-Ozkerim, Y.S. Zhang, K. Yue, K. Zhu, W. Liu, Q. Pi et al. 2016. Direct 3D bioprinting of perfusable vascular constructs using a blend bioink. *Biomaterials* 106: 58–68. doi:10.1016/j.biomaterials.2016.07.038.
- Jin, G., M.P. Prabhakaran, and S. Ramakrishna. 2011. Stem cell differentiation to epidermal lineages on electrospun nanofibrous substrates for skin tissue engineering. *Acta Biomaterialia* 7 (8): 3113–3122. doi:10.1016/j.actbio.2011.04.017.
- Johnson, F.A., D.N.Q.M. Craig, and A.D. Mercer. 1997. Characterization of the block structure and molecular weight of sodium alginates. *Journal of Pharmacy and Pharmacology* 49 (7): 639–643. doi:10.1111/j.2042-7158.1997.tb06085.x.
- Kang, H.W., S.J. Lee, I.K. Ko, C. Kengla, J.J. Yoo, and A. Atala. 2016. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*, October 2015.. doi:10.1038/nbt.3413.
- Keane, T.J., R. Londono, N.J. Turner, and S.F. Badylak. 2012. Consequences of ineffective decellularization of biologic scaffolds on the host response. *Biomaterials* 33 (6): 1771–1781. doi:10.1016/j.biomaterials.2011.10.054.
- Kesti, M., M. Müller, J. Becher, M. Schnabelrauch, M. D’Este, D. Eglin, M. Zenobi-Wong et al. 2015. A versatile bioink for three-dimensional printing of cellular scaffolds based on thermally and photo-triggered tandem gelation. *Acta Biomaterialia* 11: 162–172. doi:10.1016/j.actbio.2014.09.033.
- Khan, F., and S.R. Ahmad. 2013. Polysaccharides and their derivatives for versatile tissue engineering application. *Macromolecular Bioscience* 13 (4): 395–421. doi:10.1002/mabi.201200409.
- Khawliwala, C., R. Law, B. Shepherd, S. Dorfman, and M. Csete. 2012. 3D Cell bioprinting for regenerative medicine research and therapies. *Gene Therapy and Regulation* 1–19. doi:10.1142/S1568558611000301.
- Khor, E., and L.Y. Lim. 2003. Implantable applications of chitin and chitosan. *Biomaterials* 24 (13): 2339–2349.
- Kim, Y.B., H. Lee, and G.H. Kim. 2016. Strategy to achieve highly porous/biocompatible macroscale cell blocks, using a collagen/genipin-bioink and an optimal 3D printing process. *ACS Applied Materials & Interfaces* 8 (47): 32230–32240. doi:10.1021/acsami.6b11669.
- Kojima, K., and C.A. Vacanti. 2014. Tissue engineering in the trachea. *Anatomical Record* 297 (1): 44–50. doi:10.1002/ar.22799.
- Kolesky, D.B., K.A. Homan, M.A. Skylar-Scott, and J.A. Lewis. 2016. Three-dimensional bioprinting of thick vascularized tissues. *Proceedings of the National Academy of Sciences* 113 (12): 3179–3184. doi:10.1073/pnas.1521342113.
- Kolesky, D.B., R.L. Truby, A. Sydney Gladman, T.A. Busbee, K.A. Homan, and J.A. Lewis. 2014. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials (Deerfield Beach, Fla.)* 26 (19): 3124–3130. doi:10.1002/adma.201305506.

- Langer, R., and J. Vacanti. 2016. Advances in tissue engineering. *Journal of Pediatric Surgery* 51 (1): 8–12. doi:10.1016/j.jpedsurg.2015.10.022.
- Le Guehennec, L., E. Goyenvalle, E. Aguado, P. Pilet, R. Spaethe, and G. Daculsi. 2007. Influence of calcium chloride and aprotinin in the in vivo biological performance of a composite combining biphasic calcium phosphate granules and fibrin sealant. *Journal of Materials Science: Materials in Medicine* 18 (8): 1489–1495. doi:10.1007/s10856-006-0086-x.
- Lee, H., W. Han, H. Kim, D.H. Ha, J. Jang, B.S. Kim, and D.W. Cho. 2017. Development of liver decellularized extracellular matrix bioink for 3D cell printing-based liver tissue engineering. *Biomacromolecules*. doi:10.1021/acs.biomac.6b01908.
- Lee, J.S., J.M. Hong, J.W. Jung, J.H. Shim, J.H. Oh, and D.W. Cho. 2014b. 3D printing of composite tissue with complex shape applied to ear regeneration. *Biofabrication* 6 (2): 24103. doi:10.1088/1758-5082/6/2/024103.
- Lee, J.S., J. Shin, H.M. Park, Y.G. Kim, B.G. Kim, J.W. Oh, and S.W. Cho. 2014a. Liver extracellular matrix providing dual functions of two-dimensional substrate coating and three-dimensional injectable hydrogel platform for liver tissue engineering. *Biomacromolecules* 15 (1): 206–218. doi:10.1021/bm4015039.
- Lee, K.Y., and D.J. Mooney. 2001. Hydrogels for tissue engineering. *Chemical Reviews* 101 (7): 1869–1879. doi:10.1021/cr000108x.
- Lee, T.Y., T.M. Roper, E.S. Jonsson, I. Kudyakov, K. Viswanathan, C. Nason, C.A. Guymon, and C.E. Hoyle. 2003. The kinetics of vinyl acrylate photopolymerization. *Polymer* 44 (10): 2859–2865. doi:10.1016/S0032-3861(03)00213-1.
- Lee, Y.B., S. Polio, W. Lee, G. Dai, L. Menon, R.S. Carroll, and S.S. Yoo. 2010. Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture. *Experimental Neurology* 223 (2): 645–652. doi:10.1016/j.expneurol.2010.02.014.
- Lin, R.Z., and H.Y. Chang. 2008. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal* 3 (9–10): 1172–1184. doi:10.1002/biot.200700228.
- Liu, W., Z. Zhong, N. Hu, Y. Zhou, L. Maggio, A. Miri, A. Fragasso, X. Jin, A. Khademhosseini, and Y.S. Zhang. 2017a. Coaxial extrusion bioprinting of 3D microfibrous constructs with cell-favorable gelatin methacryloyl microenvironments. *Biofabrication*. doi:10.1088/1758-5090/aa9d44.
- Liu, X., Y. Zuo, J. Sun, Z. Guo, H. Fan, and X. Zhang. 2017b. Degradation regulated bioactive hydrogel as the bioink with desirable moldability for microfluidic biofabrication. *Carbohydrate Polymers* 178: 8–17. doi:10.1016/J.CARBPOL.2017.09.014.
- Liu Tsang, V., A.A. Chen, L.M. Cho, K.D. Jadin, R.L. Sah, S. DeLong, J.L. West, and S.N. Bhatia. 2007. Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 21 (3): 790–801. doi:10.1096/fj.06-7117com.
- Lonekera, A.E., D.M. Faulka, G.S. Hussey, A. D'Amorea, and S.F. Badylak. 2015. Solubilized liver extracellular matrix maintains primary rat hepatocyte phenotype in-vitro. *Journal of Biomedical Materials Research Part A* 104: 957–965.
- Lorson, T., S. Jaksch, M. M. Lübtow, T. Jüngst, J. Groll, T. Lühmann, and R. Luxenhofer. 2017. A thermogelling supramolecular hydrogel with sponge-like morphology as a cytocompatible bioink. *Biomacromolecules* 18 (7): 2161–2171. doi:10.1021/acs.biomac.7b00481.
- Malda, J., J. Visser, F.P. Melchels, T. Jüngst, W.E. Hennink, W.J.A. Dhert, J. Groll, and D.W. Huttmacher. 2013. 25th anniversary article: Engineering hydrogels for biofabrication. *Advanced Materials* 25 (36): 5011–5028. doi:10.1002/adma.201302042.
- Nahmias, Y., R.E. Schwartz, C.M. Verfaillie, and D.J. Odde. 2005. Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnology and Bioengineering* 92 (2): 129–136. doi:10.1002/bit.20585.

- Nakayama, K.H., C.A. Batchelder, C.I. Lee, and A.F. Tarantal. 2010. Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering. *Tissue Engineering Part A* 16 (7): 2207–2216. doi:10.1089/ten.tea.2009.0602.
- Nimmo, C.M., and M.S. Shoichet. 2011. Regenerative biomaterials that “Click”: Simple, aqueous-based protocols for hydrogel synthesis, surface immobilization, and 3D patterning. *Bioconjugate Chemistry* 22 (11): 2199–2209. doi:10.1021/bc200281k.
- Norotte, C., F.S. Marga, L.E. Niklason, and G. Forgacs. 2009. Biomaterials scaffold-free vascular tissue engineering using bioprinting. *Biomaterials* 30 (30): 5910–5917. doi:10.1016/j.biomaterials.2009.06.034.
- Ouyang, L., C.B. Highley, C.B. Rodell, W. Sun, and J.A. Burdick. 2016. 3D printing of shear-thinning hyaluronic acid hydrogels with secondary cross-linking. *ACS Biomaterials Science & Engineering* 2 (10): 1743–1751. doi:10.1021/acsbiomaterials.6b00158.
- Ouyang, L., R. Yao, S. Mao, X. Chen, J. Na, and W. Sun. 2015. Three-dimensional bioprinting of embryonic stem cells directs highly uniform embryoid body formation. *Biofabrication* 7 (4): 44101. doi:10.1088/1758-5090/7/4/044101.
- Parsa, S., M. Gupta, F. Loizeau, and K.C. Cheung. 2010. Effects of surfactant and gentle agitation on inkjet dispensing of living cells. *Biofabrication* 2 (2): 25003. doi:10.1088/1758-5082/2/2/025003.
- Pati, F., D.H. Ha, J. Jang, H.H. Han, J.W. Rhie, and D.W. Cho. 2015. Biomimetic 3D tissue printing for soft tissue regeneration. *Biomaterials* 62: 164–175. doi:10.1016/j.biomaterials.2015.05.043.
- Pati, F., J. Jang, D.H. Ha, S.W. Kim, J.W. Rhie, J.H. Shim, D.H. Kim, and D.W. Cho. 2014. Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. *Nature Communications* 5: 3935. doi:10.1038/ncomms4935.
- Peak, C.W., J. Stein, K.A. Gold, and A.K. Gaharwar. 2017. Nanoengineered colloidal inks for 3D bioprinting. *Langmuir*. doi:10.1021/acs.langmuir.7b02540.
- Pepper, M.E., V. Seshadri, T.C. Burg, K.J.L. Burg, and R.E. Groff. 2012. Characterizing the effects of cell settling on bioprinter output. *Biofabrication* 4 (1): 11001. doi:10.1088/1758-5082/4/1/011001.
- Phelps, E.A., N.O. Enemchukwu, V.F. Fiore, J.C. Sy, N. Murthy, T.A. Sulchek, T.H. Barker, and A.J. García. 2012. Maleimide cross-linked bioactive PEG hydrogel exhibits improved reaction kinetics and cross-linking for cell encapsulation and in situ delivery. *Advanced Materials* 24 (1): 64–70. doi:10.1002/adma.201103574.
- Poldervaart, M.T., H. Gremmels, K. van Deventer, J.O. Fledderus, F.C. Oner, M.C. Verhaar, W.J.A. Dhert, and J. Alblas. 2014. Prolonged presence of VEGF promotes vascularization in 3D bioprinted scaffolds with defined architecture. *Journal of Controlled Release: Official Journal of the Controlled Release Society* 184: 58–66. doi:10.1016/j.jconrel.2014.04.007.
- Rodell, C.B., N.N. Dusaj, C.B. Highley, and J.A. Burdick. 2016. Injectable and cyto-compatible tough double-network hydrogels through tandem supramolecular and covalent crosslinking. *Advanced Materials* 28 (38): 8419–8424. doi:10.1002/adma.201602268.
- Rutz, A.L., K.E. Hyland, A.E. Jakus, W.R. Burghardt, and R.N. Shah. 2015. A multimaterial bioink method for 3D printing tunable, cell-compatible hydrogels. *Advanced Materials* 1–8. doi:10.1002/adma.201405076.
- Sacchi, V., R. Mittermayr, J. Hartinger, M.M. Martino, K.M. Lorentz, S. Wolbank, A. Hofmann et al. 2014. Long-lasting fibrin matrices ensure stable and functional angiogenesis by highly tunable, sustained delivery of recombinant VEGF164. *Proceedings of the National Academy of Sciences of the United States of America* 111 (19): 6952–6957. doi:10.1073/pnas.1404605111.

- Sakai, S., H. Ohi, T. Hotta, H. Kamei, and M. Taya. 2017. Differentiation potential of human adipose stem cells bioprinted with hyaluronic acid/gelatin-based bioink through microextrusion and visible light-initiated crosslinking. *Biopolymers*, e23080. doi:10.1002/bip.23080.
- Saldin, L.T., M.C. Cramer, S.S. Velankar, L.J. White, and S.F. Badylak. 2016. Extracellular matrix hydrogels from decellularized tissues: Structure and function. *Acta Biomaterialia* 49: 1–15. doi:10.1016/j.actbio.2016.11.068.
- Shim, J.H., J.Y. Kim, M. Park, J. Park, and D.W. Cho. 2011. Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. *Biofabrication* 3. doi:10.1088/1758-5082/3/3/034102.
- Skardal, A., M. Devarasetty, H.W. Wook Kang, I. Mead, C. Bishop, T. Shupe, S.J. Lee et al. 2015. A hydrogel bioink toolkit for mimicking native tissue biochemical and mechanical properties in bioprinted tissue constructs. *Acta Biomaterialia* 25: 24–34. doi:10.1016/j.actbio.2015.07.030.
- Skardal, A., J. Zhang, and G.D. Prestwich. 2010. Bioprinting vessel-like constructs using hyaluronan hydrogels crosslinked with tetrahedral polyethylene glycol tetracylates. *Biomaterials* 31 (24): 6173–6181. doi:10.1016/j.biomaterials.2010.04.045.
- Snyder, J.E., Q. Hamid, C. Wang, R. Chang, K. Emami, H. Wu, and W. Sun. 2011. Bioprinting cell-laden matrigel for radioprotection study of liver by pro-drug conversion in a dual-tissue microfluidic chip. *Biofabrication* 3 (3): 34112. doi:10.1088/1758-5082/3/3/034112.
- Stichler, S., T. Böck, N. Paxton, S. Bertlein, R. Levato, V. Schill, W. Smolan et al. 2017. Double printing of hyaluronic acid/poly(glycidol) hybrid hydrogels with poly(ϵ -caprolactone) for MSC chondrogenesis. *Biofabrication* 9 (4): 44108. doi:10.1088/1758-5090/aa8cb7.
- Syedain, Z.H., J. Bjork, L. Sando, and R.T. Tranquillo. 2009. Controlled compaction with ruthenium-catalyzed photochemical cross-linking of fibrin-based engineered connective tissue. *Biomaterials* 30 (35): 6695–6701. doi:10.1016/j.biomaterials.2009.08.039.
- Tan, Y., D.J. Richards, T.C. Trusk, R.P. Visconti, M.J. Yost, M.S. Kindy, C.J. Drake, W.S. Argraves, R.R. Markwald, and Y. Mei. 2014. 3D printing facilitated scaffold-free tissue unit fabrication. *Biofabrication* 6 (2): 24111. doi:10.1088/1758-5082/6/2/024111.
- Wang, X., Y. Yan, Y. Pan, Z. Xiong, H. Liu, J. Cheng, F. Liu et al. 2006. Generation of three-dimensional hepatocyte/gelatin structures with rapid prototyping system. *Tissue Engineering* 12 (1): 83–90. doi:10.1089/ten.2006.12.ft-16.
- Yeo, M.G., and G.H. Kim. 2017. A cell-printing approach for obtaining hASC-laden scaffolds by using a collagen/polyphenol bioink. *Biofabrication* 9 (2): 25004. doi:10.1088/1758-5090/aa6997.
- Yeo, M., J.S. Lee, W. Chun, and G.H. Kim. 2016. An innovative collagen-based cell-printing method for obtaining human adipose stem cell-laden structures consisting of core–sheath structures for tissue engineering. *Biomacromolecules* 17 (4): 1365–1375. doi:10.1021/acs.biomac.5b01764.
- Zheng, M., L. Zheng, P. Zhang, J. Li, and Y. Zhang. 2015. Development of bioorthogonal reactions and their applications in bioconjugation. *Molecules* 20 (2): 3190–3205. doi:10.3390/molecules20023190.
- Zhu, J. 2010. Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials* 31 (17): 4639–4656. doi:10.1016/j.biomaterials.2010.02.044.

Chapter 3 Applications of extrusion bioprinting *Past, present, future*

G. Forgacs, F. Marga, and K. Jakab

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3.1 Introduction

Bioprinting has seen spectacular progress in recent years, as can be judged by the steadily increasing number of publications and professional meetings devoted to the topic or by the number of google-search entries on the subject. Numerous reviews have been devoted lately to the general overview of the field (Arslan-Yildiz et al., 2016; Cui et al., 2017; Ferris et al., 2013; Li et al., 2016; Ozbolat et al., 2016; Pati et al., 2016; Seol et al., 2014; Skardal and Atala, 2015) or its applications in many specialized domains (Albritton and Miller, 2017; Datta et al., 2017;

Di Bella et al., 2015; Dias et al., 2014; Duan, 2017; Hoch et al., 2014; Irvine and Venkatraman, 2016; Jana and Lerman, 2015; Knowlton et al., 2015; Ng et al., 2016; Tasoglu and Demirci, 2013; Vijayavenkataraman et al., 2016; Visscher et al., 2016; Zhang et al., 2016), as discussed in the present volume also. With the appearance of commercial entities (e.g., Organovo [www.organovo.com], Cyfuse [<http://www.cyfusebio.com/en/>], Poietis [<http://poietis.com>]), the field has matured beyond basic academic research.

Many of the reviews on the subject touch upon the distinct technologies that are so far employed, the major ones being inkjet, extrusion, laser, and acoustic bioprinting (Ali et al., 2014; Devillard et al., 2014; Gudapati et al., 2016; Ng et al., 2017; Ozbolat and Hospodiuk, 2016; Seol et al., 2014). These various technologies differ in the type of bioink units used and the spatial resolution achieved. The term bioink (detailed in [Chapter 2](#)) refers to the object the printer deposits and may be composed of entirely living (i.e., cellular) or entirely nonliving material (e.g., hydrogels, polymers for the building of scaffolds, specialized materials for the manufacturing of implants and prosthetics) or hybrids (i.e., cell-laden hydrogels).

The modern era of bioprinting started with the work of Thomas Boland, who was the first to deposit living cells (proteins and bacteria originally, Wilson and Boland, 2003) with a simple desktop inkjet printer reengineered to print not only in the plane but also in the z direction. In inkjet bioprinting the bioink units are liquid or polymer droplets of 50–300 μm ejected from the printer by means of piezo or thermal transducer. Inkjet bioprinting was soon followed by extrusion bioprinting. This technology is capable of manipulating robust bioink units in the form of multicellular spheroids or other types of minitissues (Jakab et al., 2004; Neagu et al., 2005; Norotte et al., 2009) that are extruded typically by pressure-operated valves or mechanically driven pistons. The typical resolution that can be achieved (e.g., determined by the diameter of the extruded spheroid) is 200–500 micron. Laser-assisted bioprinting (Guillemot et al., 2010a, 2010b; Guillotin et al., 2010; Ringeisen et al., 2004) operates with bioinks of cellular dimensions (~ 10 micron). In acoustic bioprinting, the bioink (with linear dimensions of 10–500 μm is ejected by an acoustic wave (Demirci and Montesano, 2007). Even though the four technologies mentioned here are popular among the practitioners of the field, extrusion bioprinting has gained particular attention in the past years. In this chapter, we will discuss extrusion bioprinting as applied to the deposition of cellular (i.e., living) material.

In what follows, we will first briefly overview the major components of extrusion bioprinting and its early history. We will then present examples of the applications of the technology to diverse fields, such as embryonic development, tissue and organ engineering, and drug development and testing. We will devote considerable part of this chapter to the discussion of our views, shared by many

colleagues, on the future of bioprinting. We will first discuss potential applications, which are, in some way already in the pipeline, followed by more distant but still rational scenarios. In the course of our presentation we will make every effort to mitigate the hype still surrounding the field and emphasize its realistic promise.

3.2 Major components of extrusion-based bioprinting

The bioprinting process, when it involves living, cellular material (as will be the case discussed in this chapter), fundamentally differs from the printing of inanimate structures. It is composed of three well-defined phases, each critical to the success of the final outcome: pre-processing, processing, and post-processing (Marga et al., 2012).

3.2.1 Pre-processing

Pre-processing is the phase in which the bioink units are prepared. This could be a highly nontrivial step, as evident when the bioink units are multicellular spheroids. Making such spheroids expeditiously and reproducibly with uniform size is a complex task (Itoh et al., 2015; Lin and Chang, 2008; Mironov et al., 2009; Napolitano et al., 2007; Norotte et al., 2009). The composition of the bioink depends on what is the desired tissue or organ structure to be built. If the goal is to prepare liver tissue the bioink should contain hepatocytes and possibly other hepatic cells (e.g., stellate cells, Kupffer cells). In the case of a vascular graft the bioink should be composed of endothelial cells (EC), smooth muscle cells (SMC), and possibly fibroblasts (FB). Thus the term *universal bioink*, as sometimes referred to lately, is misleading and simply an oxymoron.

The challenge in preparing cellular aggregates to be dispensed by an extrusion device lies in the requirements of uniform size and shape to fit these units into the printer cartridge. Several methods have been developed depending on the type of cells employed and the shape of the units considered (Lin and Chang, 2008).

Cellular spheroids: When cells possess cell–cell adhesion molecules, it is sufficient to place a single-cell solution in a nonadhesive environment and to rely on several aspects of the liquid-like nature of cells and tissues (Kosztin et al., 2012; Steinberg, 2007). Droplets of a single-cell suspension in cell culture medium can be dispensed to form hanging drops, where, under physiological conditions, cells coalesce at the medium-air interface and form cell spheroids (Foty, 2011). The same cell solution pipetted over an array of millimeter-sized wells stamped into a nonadhesive hydrogel (such as agarose) leads to the same result (Figure 3.1) In both methods the size of the spheroids is tunable through

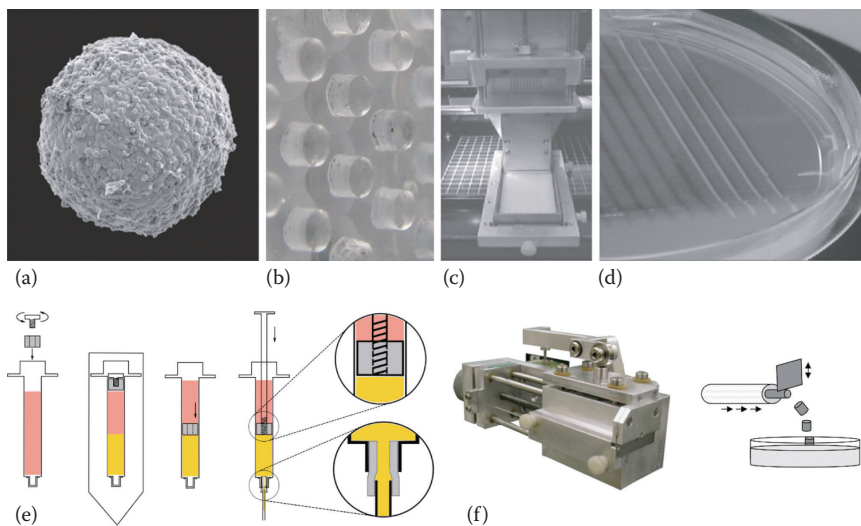


Figure 3.1 (a) SEM image of a spherical, 500 μm cellular spheroid composed of Chinese hamster ovary cells. (b) Stamp used to create microwell arrays. (c) Device to extrude cellular cylinders into nonadhesive agarose grooves shown in panel (d). (e) Filling capillaries with cellular pellets. Magenta and yellow colors refer, respectively, to cell solution or supernatant and cellular pellet as a result of centrifugation. (f) Preparation of cylinders of equal diameter and height. The device holds a capillary filled with the cell pellet. It is automated to extrude a cellular cylinder to be chopped with a blade and subsequently advanced. Panels a, c, and d from Norotte et al. (2009).

the cell concentration. These methods are suitable to create distinct microstructures within a spheroid: mixing two or more cell types with different cohesive properties leads to cell sorting, where a sphere-within-a-sphere forms (Foty et al., 1996).

Cellular cylinders: For the preparation of cylindrical bioink, the cell solution is first concentrated by centrifugation and the resulting cell pellet is subsequently aspirated into a capillary tube serving as a bioink cartridge (Figure 3.1). Following a brief incubation time, cellular cylinders can be dispensed from the cartridges directly into a construct or extruded into nonadhesive grooves for further maturation, where they gain structural integrity (Marga et al., 2012; Norotte et al., 2009).

A particular invention employs such prefabricated cylinders to prepare uniform cellular spheroids: The cylinders are cut into smaller ones with equal diameters and heights that can be either readily used or further matured to become spherical (Figure 3.1).

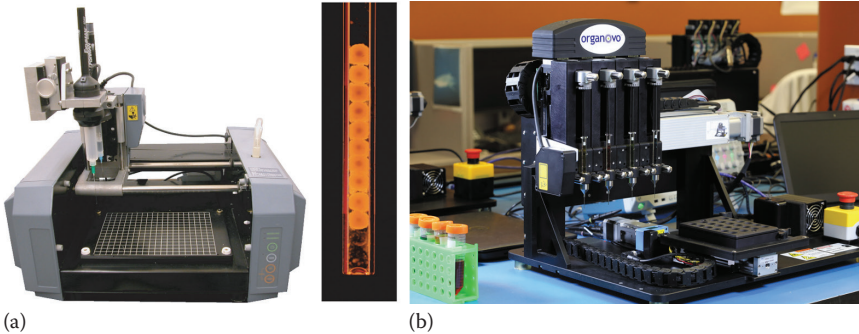


Figure 3.2 (a) The first extrusion bioprinter, fabricated by NeatCo (Carlisle, Canada; modified at the University of Missouri-Columbia to accommodate two printing heads), this printer is used to build extended living structures and its microcapillary printer cartridge (right) (inner diameter of 500 μm) is filled with spheroidal minitissue bioink particles. (b) Organovo's four-headed bioprinter currently in use. (From Neagu, A. et al., *Phys. Rev. Lett.*, 95, 178104, 2005.)

3.2.2 Processing

Processing refers to the actual deposition of the bioink units by the bioprinter (Figure 3.2). There is nothing magic in these machines; they are tools that make the deposition more accurate, reproducible, and fast. Contrary to the printing with nonliving material the bioprinting process itself does not result in a useful product. It merely assures the reproducible and timely arrangement of the bioink particles.

A typical extrusion bioprinter has two or more printing heads capable of extruding either cellular bioink particles or biocompatible gels. Its cartridges are syringes, capillaries, or needles and extrusion is accomplished by pistons or via hydraulic pressure. The main advantage of the relatively low speed and/or pressure extrusion lies in circumventing the harsh conditions (shear, shock, heat, etc.) that the cells may encounter in other printing approaches. Currently, several commercial suppliers offer bioprinters (e.g., Envisiontec, Regenovo).

3.2.3 Post-processing

It is only in the post-processing phase that through morphogenetic mechanisms akin to those acting in early embryonic development (e.g., tissue fusion and cell sorting [Perez-Pomares and Foty, 2006]) the printed set of discrete bioink units matures into the final functional continuous biological structure.

The printed constructs may contain permanent scaffolding material that integrates with the tissue, preserves its structural integrity, or adds a desired functionality. Transient scaffolds are used to stabilize the initial 3D configuration.

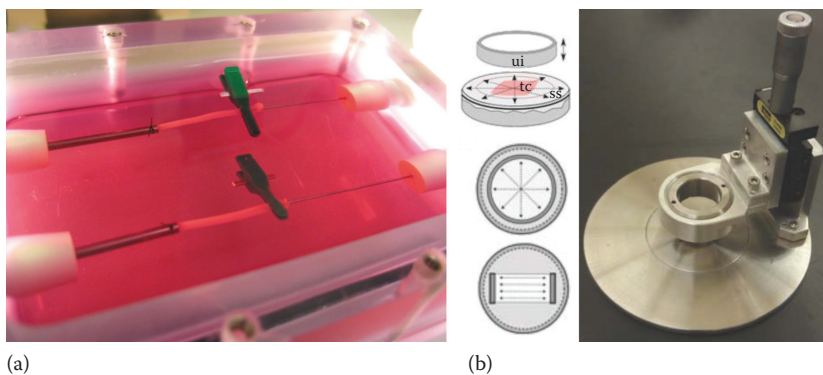


Figure 3.3 (a) Bioprinted vascular grafts in a perfusion bioreactor designed to exert periodic radial strain through pulsatile flow to mimic physiological conditions during post-printing maturation. (From Norotte, C. et al., *Biomaterials*, 30, 5910–5917, 2009.) (b) Schematic illustration of a bioprinted smooth muscle construct cultured on a cell-adhesive elastic-membrane bioreactor. The ring assembly is inserted into the shown device that produces an indentation in the membrane resulting in radial or longitudinal strain necessary for the proper maturation of the muscle construct.

Once the bioink units are fused, the transient scaffold is removed either by chemical treatment (enzymatic treatment, change in ionic strength, pH) or physical treatment (change in temperature, mechanical action) and the stabilized construct is transferred into the specialized bioreactors for further maturation (Figure 3.3).

The useful biological structure forms during the post-printing maturation. It is only through this process that the cellular assembly turns into an implantable tissue or organ structure such as a vascular conduit with the proper burst pressure, contractile properties, and suture retention strength.

3.3 The past: Early applications

3.3.1 Rings and sheets

The initial proof of concept study on extrusion bioprinting involved the deposition of a monolayer of spheroids. Figure 3.4 illustrates the printing and the postprinting configurations of a ring (Jakab et al., 2004) and a sheet (Jakab et al., 2008). For the ring, 10 spheroids composed of Chinese hamster ovary (CHO) cells were deposited in a collagen gel, itself printed. The collagen had to be printed as a cold liquid solution but it rapidly and sufficiently gelled for the spheroids to remain in the defined pattern. The spheroids were deposited in close proximity to induce

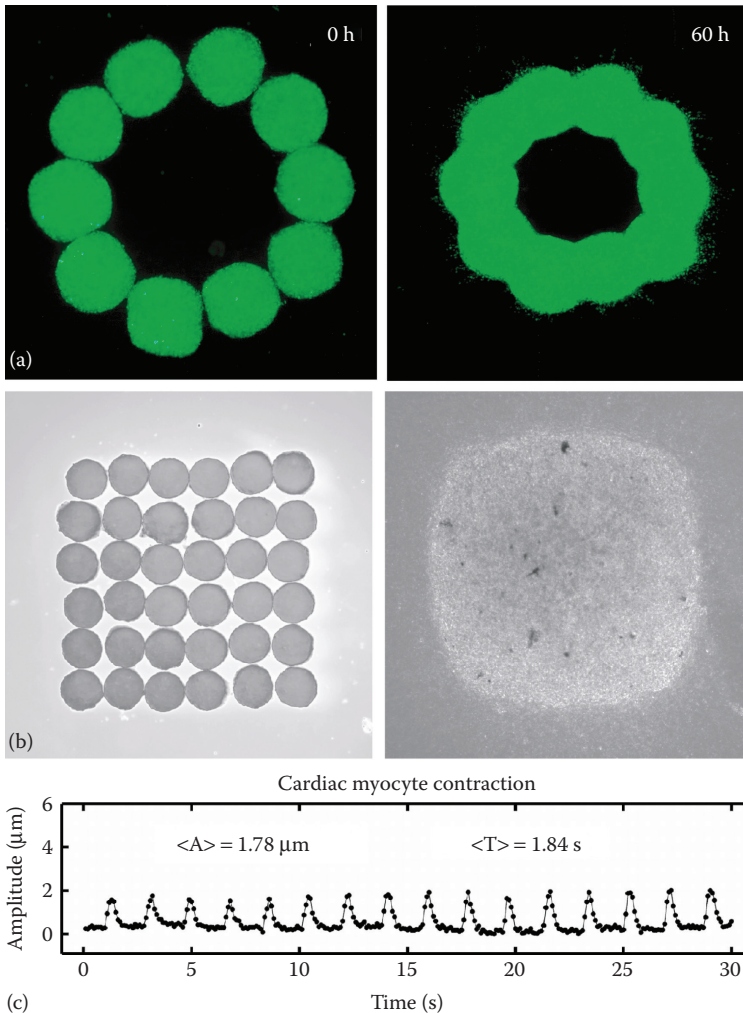


Figure 3.4 Extrusion printing of cellular structures with defined topology. (a) Ring: Initial configuration (left) and fused pattern (right), 60 h post-printing of spheroidal bioink particles composed of CHO cells deposited into 1 mg/mL collagen type 1 gel. (b) Sheet: Building a pulsatile cardiac construct with bioink particles composed of chicken embryonic cardiomyocytes (left) printed into 1 mg/mL collagen type 1 gel. The fused pattern (right) forms in about 70 h. (c) The synchronous beating of the fused construct in (b). $\langle A \rangle$ and $\langle T \rangle$ are the amplitude and period of beating, respectively. All bioink particles in this figure are of average 500 μm diameter. Panel (a) (From Jakab, K. et al., *PNAS*, 101, 2864–2869, 2004. With permission.), panels (b) and (c) (From Jakab, K. et al., *Tissue Eng. Part A.*, 14, 413–421, 2008. With permission.)

cell adhesion and fusion, leading to the formation of a ring. As fusion progressed, the cells rearranged the surrounding matrix by pulling on the collagen fibers resulting in the narrowing of the ring's lumen (Figure 3.4a).

For the sheet configuration (also printed into collagen, Figure 3.4b) the bioink was composed of chicken embryonic cardiomyocytes. This latter example not only demonstrated the formation of a coherent tissue through fusion but, importantly, the preservation of the cells' functionality, as shown by the resulting beating cardiac patch (Figure 3.4c).

3.3.2 Tubular structures: Vascular and nerve grafts

Evolution of extrusion bioprinting for tubular structures: The first attempt to print tubular structures was based on the method described for the rings and sheets earlier. A first ring of spheroids was printed into a collagen gel layer. Next, another layer of collagen was deposited on top and a new ring of spheroids was printed and the process continued. The initial configuration of a three-ring construct and the ensuing fusion at 12 h and 24 h post printing are shown in Figure 3.5. Success of forming a tubular structure depends on several factors. First, the subsequent collagen layers need to be consistent: If they were too liquid or too gelled, the spheroids in the different layers did not align, leading to defects in the tubular structure (as seen on the top-right section of the tube in Figure 3.5). Second, printing rings accurately on top of each other turned out to be challenging, thus making the process cumbersome and prone to error. In addition, with an increase in the size the manual preparation of spheroids became a severely rate-limiting step.

The limitations were countered by the gradual improvement of the process: Collagen layers were replaced with agarose rods; an

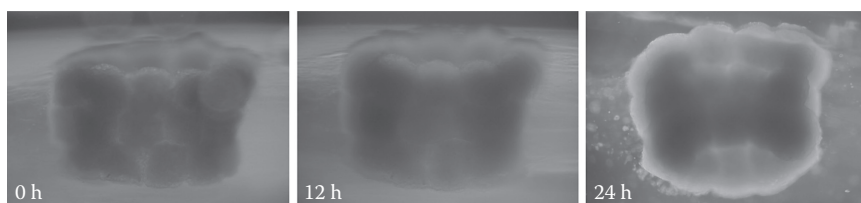


Figure 3.5 Time evolution of a vertically printed three-layered tubular structure made with spherical bioink particles composed of CHO cells. (From Jakab, K. et al., *Tissue Eng. Part A*, 14, 413–421, 2008. With permission.)

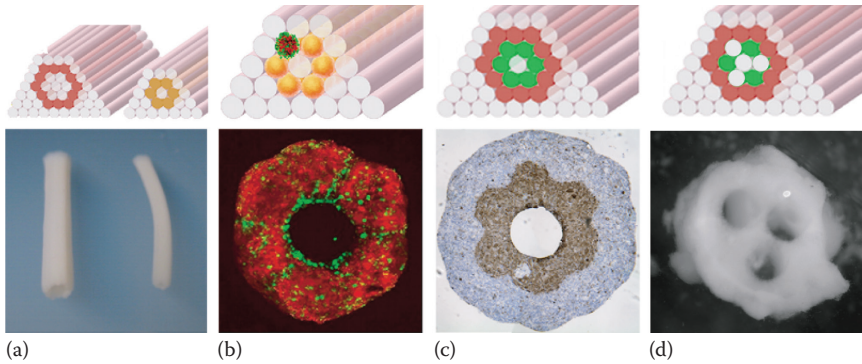


Figure 3.6 Design templates for the bioprinting of tubular structures (top) and the corresponding fused constructs (bottom). (a) Tubes of different diameters. Colored circles denote the cellular bioink units, pink rods designate the agarose support. (b) Construct built with spheroids composed of a mixture of SMC (red) and EC (green). A cross-section of the fused tube (bottom) shows that most ECs migrated toward the lumen. (c) Double-layered vascular tube. The inner layer is made of SMC building blocks, the outer one of FB building blocks. The cross-section (bottom) shows that the building blocks fused but the two cell types (the inner SMCs are immune-stained for actin in brown) remained segregated mimicking the media and adventitia of blood vessels. (d) Design template for a multichannel tube used as a nerve graft. The external wall is built with units composed of bone marrow stem cells (red). The resulting lumen (bottom) is divided into three individual channels after fusion of the three cellular rods of heterocellular composition (BMSC and SCs; green) placed alternatively with agarose rods (removed after fusion of the bioink units). (From Jakab, K., et al., *Biofabrication*, 2, 022001, 2010.)

automated spheroid preparation method was first devised; and, later, the spheroids were replaced by cellular rods. Design templates for the improved process are shown in [Figure 3.6](#) and were used for the layer-by-layer printing of the bioink and agarose rods. Post deposition the agarose rods (to which cells did not adhere) were mechanically removed. Size of the tubular lumen was easily modified either by using different diameters for the printed units (200 to 500 μm) or more units ([Figure 3.6a](#)). The number of lumens could also be varied as shown in [Figure 3.6d](#).

Vascular and nerve graft: From cellular composition to maturation:

The following examples were constructed to respond to two objectives: (1) provide a solution to the lack of functional small diameter

vascular grafts (Norotte et al., 2009) and (2) create a fully biological nerve graft for peripheral nerve repair (Marga et al., 2012; Owens et al., 2013). Selection of cells for the bioink was critical to improve functionality of the bioprinted constructs. EC are crucial to the proper functioning of a blood vessel. Thus first spheroids composed of a mixture of SMC and EC were used to form a vascular graft (Figure 3.6b). After fusion, the ECs sorted and formed the necessary continuous layer lining the lumen. To further approximate true blood vessels, FB were added to form a layer next to the SMC layer (Figure 3.6c). After fusion the SMC and FB layers remained separated and the composition with the three cell types resembled the structure of blood vessels. About 6 h post-printing the agarose support was removed and the conduit was allowed to further mature in a perfusion bioreactor for up to 21 days. Such maturation step was necessary for the production of extracellular matrix and it enhanced the mechanical strength as demonstrated by the gradual increase in burst pressure (up to 773 mmHg) (Marga et al., 2012).

For the nerve graft (Marga et al., 2012; Owens et al., 2013), two cell types were used to form the bioink: (1) bone marrow stem cells (BMSC) and (2) Schwann cells (Figure 3.6d). BMSC were chosen because they can differentiate into Schwann-like cells. After fusion, a continuous tube with three longitudinal parallel internal channels was formed (the structure resembling the fascicles in true nerves). Seven days post-printing maturation the construct was used (i.e., implanted) as a nerve conduit in a rat sciatic nerve injury model to bridge the 1 cm gap between the proximal and distal ends of the severed nerve (Owens et al., 2013). The Schwann cells lined the lumens of the channels to attract and guide axons and to favor regeneration. Observation of regenerated axons through the graft after a short-term implantation (Marga et al., 2012) was followed by electrophysiological studies to evaluate sensory and motor recoveries. The bioprinted nerve graft performed better than an empty collagen nerve conduit often used in clinical settings (Owens et al., 2013).

Branched constructs: A first step toward large organs: The vascular and nerve grafts are straight tubes. They could be used to replace a short fragment of the vasculature or of a nerve. However, the formation of larger constructs/organs, the holy grail of tissue engineering, will require a vascular and nerve tree for nutrient delivery and innervation. Such endeavor depends on the possibility of building branching structures. Branching structures were also built by the outlined extrusion bioprinting technology ([Norotte et al., 2009]; Figure 3.7). Spheroids were deposited layer-by-layer (Figure 3.7a). Branches had different external diameters of 0.9 and 1.3 mm.

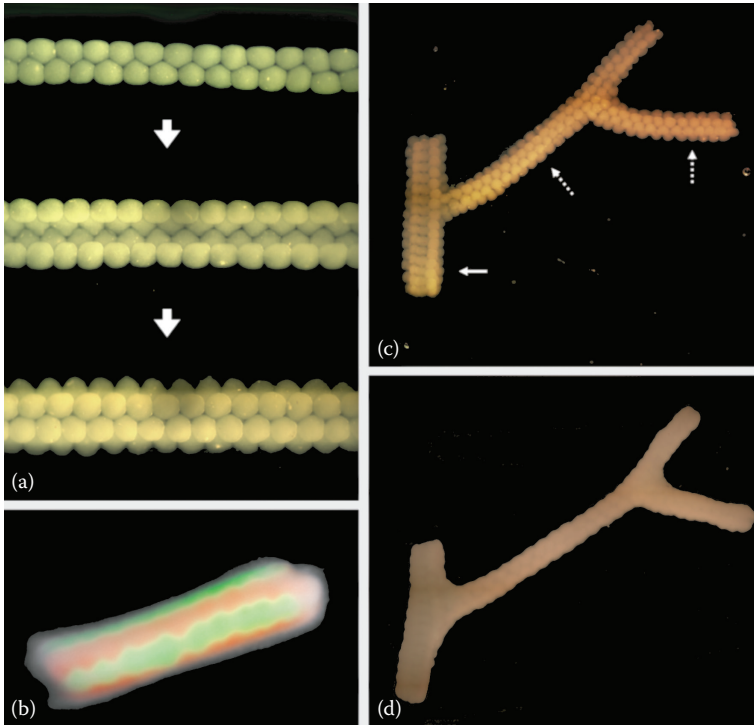


Figure 3.7 Fusion of multicellular spheroids assembled into tubular structures. (a) FB spheroids assembled according to the template in [Figure 3.6a](#). (b) Fusion pattern after 7 days post-printing of a tube assembled from fluorescently labeled red and green sequences of CHO spheroids. (c) Branched structure built of 300 μm FB spheroids with branches of 1.2 mm (solid arrow) and 0.9 mm (broken arrows). (d) The fused branched construct after 6 days post-deposition. (From Norotte, C. et al., *Biomaterials*, 30, 5910–5917, 2009.)

3.4 The present

3.4.1 Applications for basic research

In vivo, cells reside in a 3D environment: Epithelial cells are surrounded by their immediate neighbors and mesenchymal cells are surrounded by the extracellular matrix they secrete. These essential conditions are not provided in traditional 2D cell culture in Petri dishes where the cells' membrane interfaces nonphysiological partners such as the culture medium itself or other cells along essentially a line. The development of 3D cell culture methods has allowed the study of cellular interactions in physiologically more realistic environments (Justice et al., 2009;

Knight and Przyborski, 2015; Ravi et al., 2015; van Duinen et al., 2015). Bioprinting in general and extrusion bioprinting in particular are optimal tools to create 3D cell culture assays at a scale comparable to that of tissues instead of that of the cells. The typical application of extrusion bioprinting in basic research at present is to build tissue constructs for 3D cell culture to study developmental processes (e.g., early morphogenesis), cellular interactions (e.g., cell adhesion), cell activity (e.g., cell migration), and disease evolution (e.g., tumor growth). In what follows we provide a few examples of such applications.

Model for early development: Self-organization is the hallmark of embryonic development. As cells in the embryo divide and progressively reach their terminally differentiated state through interactions with their environment, they autonomously (i.e., without the interference of external agents [Whitesides and Grzybowski, 2002]) self-assemble into complex, hierarchically arranged compartmentalized structures, such as aggregates, tissues, and eventually organs (Forgacs and Newman, 2005). Examples of fundamental processes that act during this early morphogenesis are cell sorting and tissue fusion (Perez-Pomares and Foty, 2006). Extrusion bioprinting is being used extensively to study such processes and employ them in building complex biological structures. Jakab and coworkers (Jakab et al., 2004, 2008; Neagu et al., 2005) and Shafiee and coworkers (2015) printed multicellular assemblies that were used to demonstrate that tissue fusion is analogous to the coalescence of liquid drops (Figure 3.8; (Kosztin et al., 2012; Steinberg, 2007)).

The quantitative description of the fusing pattern in Figure 3.8 is provided in terms of quantities used in the physics of liquids, such as surface and interfacial tension (Foty et al., 1996; Kosztin et al., 2012) and viscosity (Forgacs et al., 1998). The notion of tissue liquidity stipulates that the minitissue spheroids in Figure 3.8 are analogous to liquid drops (Forgacs et al., 1998; Phillips and Steinberg, 1978). Differential adhesion (Steinberg, 2007) relates the effective surface tension characterizing these liquid drop analogs to molecular quantities fundamental to cellular interactions. Indeed, the bioink units shown in Figure 3.8 are composed of several thousands of CHO cells transfected with the cell adhesion molecule N-cadherin and the measurable effective surface tension characterizing these tissue liquids can be related to the number of N-cadherins on the surface of the individual CHO cells (Foty and Steinberg, 2005).

Models for the in situ monitoring of cellular processes: Cellular activity is typically observed from the *outside* by either quantifying the secretion of metabolites into the culture medium or by measuring physical quantities characterizing the cells' behavior (e.g., contraction in the case of muscle and action potential in the case of a nerve). However, such

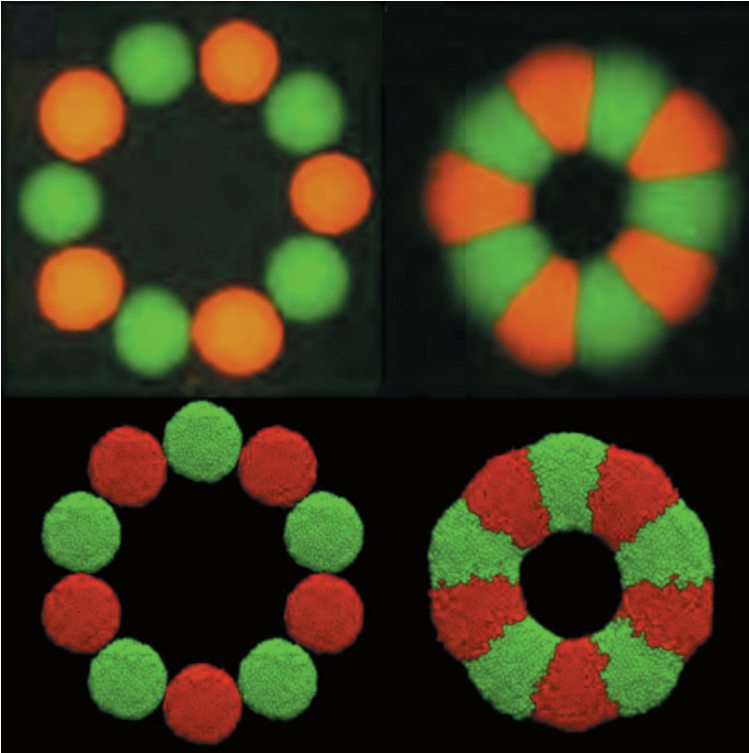


Figure 3.8 Cellular torus built with CHO cells. Top and bottom rows show, respectively, the result of bioprinting with cellular spheroids of 500 μm diameter bioink (Jakab et al., 2008) and cellular particle dynamics simulation (CPD; [Kosztin et al., 2012]). CPD simulations implement theoretical considerations from fluid dynamics. The left and right columns correspond to, respectively, the initial and final configurations. Neighboring spheroids are identical and are labeled with different colors only for better illustration. CPD simulations accurately reproduce the time evolution of the experimental configuration as the minitissue spheroids fuse. (From Kosztin, I. et al., *Rev. Mod. Phys.*, 84, 1791–1805, 2012. With permission.)

observations cannot accurately reflect how the same cells work *in vivo*. Bioprinting offers the unique opportunity to build a *lab-in-the-tissue* for the *in situ* monitoring of metabolic and functional properties of engineered tissue and organ structures.

Example 1: Serum albumin analysis in 3D-printed liver: Albumin is the most abundant serum protein in the blood. It is produced by hepatocytes in the liver and secreted into sinusoids that are specialized capillary blood vessels with fenestrated endothelium. The monitoring of albumin secretion is thus an effective way to evaluate the health of the liver

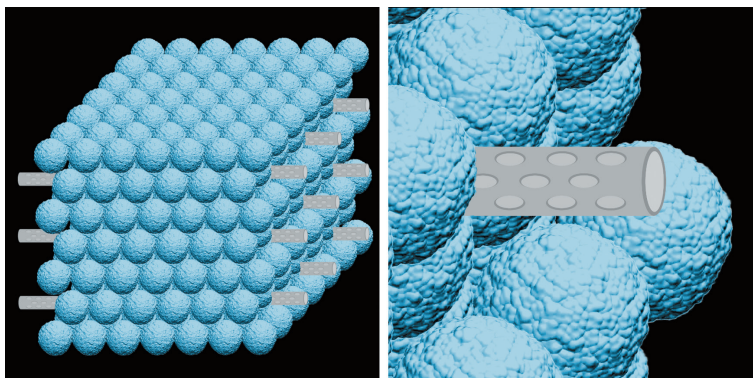


Figure 3.9 A possible realization of a lab-in-the-tissue device. The blue spheres represent hepatocyte bioink units (individual cells or aggregates), whereas the gray-perforated tubes represent microporous hollow fibers into which metabolites, such as albumin, secreted by cells deep within the tissue, can be collected.

(Nguyen et al., 2016). The schematic illustration of a possible lab-in-the-tissue device to measure albumin secretion *in situ* in a 3D-printed liver construct is shown schematically in [Figure 3.9](#).

All ingredients necessary to construct such a device are available today. Complex liver tissue containing both parenchymal cells (i.e., hepatocytes) and stromal cells (endothelial and stellate cells) has been extrusion bioprinted by the company Organovo (www.organovo.com). (Such tissues are commercially available for a number of applications; see discussion in [Section 3.4.2](#).) Microporous hollow fibers mimicking the fenestrated endothelium have been used for multiple applications and are often employed in bioreactors for tissue engineering. In particular, we refer to the work of Curcio and coworkers (2005). These authors fabricated biocompatible microporous hollow fibers with inner diameter of 200–300 μm and pore size of 200 nm and used these fibers to measure the transport across their walls of various metabolites, in particular of bovine serum albumin with a molecular weight of 67 kDa (human albumin's molecular weight is 66.5 kDa), with a Stokes diameter of 3.61 nm (the Stokes radius of a solute is the radius of a hard sphere that diffuses at the same rate as the solute). With these parts at hand, the construction of a lab-in-the-tissue device to measure albumin secretion by a (model) liver tissue would proceed as suggested by [Figure 3.9](#). Layer-by-layer deposition of the bioink (containing the mixture of liver-specific cells) by the bioprinter would be temporarily interrupted at regular time intervals to insert the hollow fibers in order to finally arrive at the structure shown in [Figure 3.9](#). Upon fusion of the discrete bioink particles, secretion of albumin into the collecting hollow

fibers will then characterize the activity of cells deep in the tissue constantly interacting with other cells, contrary to the situation when albumin is collected from the surrounding culture medium, in which case it originates mostly from the cells in the vicinity of the construct's surface.

Example 2: Monitoring contractile stress inside the cardiac tissue: Just as in the case discussed in Example 1, all the necessary ingredients for the continuous *in situ* monitoring of the contractile properties of 3D-printed cardiac tissues are in place. This can be achieved by the integration of the nanowire nanoelectronic scaffold (nanoES) developed by Tian et al. (2012) into a 3D-printed cardiac tissue construct (such as the one built for example by Jakab et al. (2008)). The device and its construction are schematically shown in Figure 3.10.

Tian et al. (2012) built nanoES within which the field emission transistor (FET) devices spanned separations of several microns to hundreds of microns, thus compatible with cellular dimensions. The authors then combined their nanowire nanoelectronic scaffolds with scaffolds built of synthetic or natural biomaterials customarily used in scaffold-based tissue engineering (Langer and Vacanti, 1993). Subsequently, they seeded the combined nanoelectronic-biomaterial scaffolds with cardiomyocytes and measured their electrical activity by recording the changes in the conductivity and electrical sensitivity of the FETs. (For the detailed manufacturing of nanoFET and their use to measure the electrical activity of cardiomyocytes, see Cohen-Karni et al., 2009.) A lab-in-the-tissue device to monitor the real-time local activity of cardiomyocytes could be built similar to the scheme outlined in Example 1. The extrusion bioprinting of bioink units composed of cardiomyocytes would be now temporarily interrupted by the insertion of the nanoES scaffolds, as suggested by the right panel in Figure 3.10.

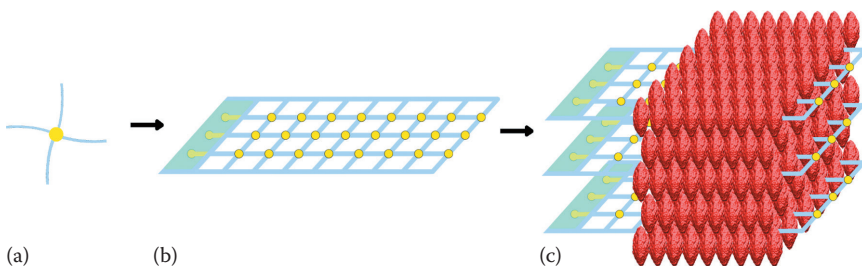


Figure 3.10 Schematic illustration of integrating nanoelectronics with 3D-printed tissue. The nanowires (yellow dots in [a]) are made into a nanowire field emission transistor (a) and these are combined into a nanoES (b). The nanoES is integrated into the layer-by-layer printing of a cardiac muscle tissue ([c]; red symbols represent cardiomyocyte bioink units, either individual cells or aggregates).

Models for cancer biology: Cancer biology has been and will remain a topic of intense research. It has long been recognized that to understand how the malignant cellular transformations develop, considering the full 3D environment of the transformed cells is necessary. Thus cellular spheroid models have been extensively used from early on to model tumor evolution (Hamilton, 1998; Kunz-Schughart et al., 1998). Then it is not surprising that bioprinting found an important application in cancer research (Albritton and Miller, 2017; Tasoglu and Demirci, 2013; Zhang et al., 2016). Due to its versatility the technology is able to respond to the demand of creating the complex 3D environment needed to study tumor models in physiologically more relevant conditions than in 2D.

A good example of such strategy is the work of Zhao et al. (2014). These authors used bioprinting to construct a 3D cervical tumor model. HeLa cells were mixed into a hydrogel composed of fibrinogen, gelatine, and alginate and the resulting bioink was deposited with an extrusion bioprinter into a grid-like structure (Figure 3.11). The printed construct was subsequently immersed into thrombin to cross-link fibrinogen. Along with the 3D construct, for comparative study, a 2D model was also prepared by seeding HeLa cells onto a Petri dish filled with cell culture medium. The results shown in Figure 3.11 illustrate the advantages of the 3D tumor model well when compared with a nonphysiological 2D cell culture assay.

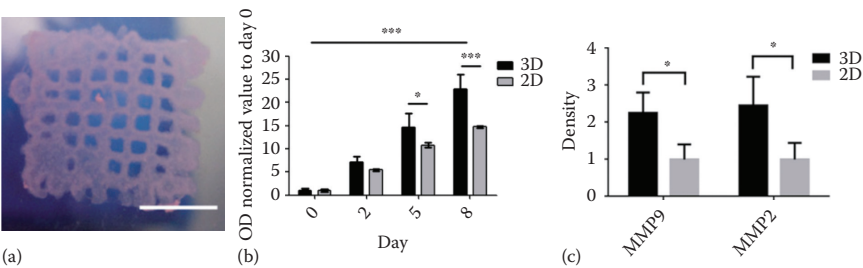


Figure 3.11 Bioprinting of a tumor model. (a) The bioprinted 3D HeLa/hydrogel construct of dimensions $10 \times 10 \times 2 \text{ mm}^3$, on day 8 post-printing. The grid-like structure, possible to achieve with the printer, assures that nutrients can reach most of the cells in the construct (scale bar $200 \mu\text{m}$). (b) Cell proliferation, evaluated by the optical density (OD) of metabolites produced by live cells, is considerably more intense in 3D (i.e., in the tumor model) than 2D. (c) The 3D hydrogel extracellular matrix (ECM)-mimic microenvironment in the tumor model favors the expression of matrix metalloproteinases, MMP-2, and MMP-9. (From Zhao, Y. et al., *Biofabrication*, 6, 035001, 2014.)

3.4.2 Applications in pharmaceuticals

Developmental drugs are first tried in cell culture assays. A false positive outcome here eventually leads to substantial delay and financial loss. Thus, probing the drug early under as realistic conditions as possible is of critical importance. Bioprinting is revolutionizing drug development. The commercially available Exvive Human Liver Tissue Model (EHLTM) by Organovo (Nguyen et al., 2016) is progressively being used in drug toxicity assays. EHLTM is a 3D human liver construct composed of hepatocytes, stellate cells, and EC. The power of EHLTM is well illustrated by the ability of this bioprinted tissue to detect toxicity where it was not observed earlier by traditional methods.

Trovafloracin is a drug that was withdrawn from the market one year after its approval as it resulted in death in a small population of patients. Its toxicity was not identified in preclinical trials. Using EHLTM, it was possible to demonstrate Trovafloracin's hepatotoxic effect at doses, at which in 2D assays it was not observed (Figure 3.12a). Furthermore, EHLTM could clearly distinguish between Trovafloracin and its molecularly closely related nontoxic variant (Figure 3.12b).

Besides EHLTM, Organovo has recently commercialized the Exvive Proximal Kidney Tissue Model as well (King et al., 2017).

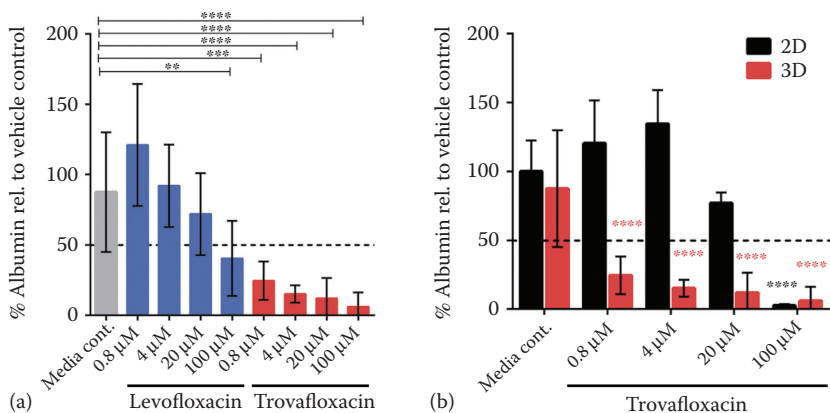


Figure 3.12 Hepatotoxicity detected by the EHLTM. (a) Measurement of dose-dependent albumin secretion, normalized to a control vehicle, in the supernatant by EHLTM treated with Trovafloracin or Levofloxacin for 7 days. The vehicle control is prepared by diluting the drugs in culture medium. (Note that the measurement of albumin in the assay proposed in Section 3.4.1 would be more accurate.) (b) Albumin secretion by EHLTM compared to that from a standard 2D hepatocyte culture treated with Trovafloracin for 7 days. Data are normalized to a vehicle control. Toxicity in both panels is defined as lower than 50% secretion in the vehicle control. (From Nguyen, D.G. et al., *PLoS One*, 11, e0158674, 2016.)

3.4.3 Toward therapeutic applications

As of today, no full-size human solid organ has been built by either bioprinting or any other organ engineering method. One reason for this is that such an organ would need complex vasculature that still represents a serious challenge for tissue engineers. Nevertheless thick vascularized tissues (Kolesky et al., 2014, 2016) and parts of organs have been successfully built recently by extrusion bioprinting and implanted into animal models, hinting at the possibility of the not-too distant therapeutic applications of bioprinting. An instructive example is the skeletal muscle constructed by Kang and coworkers (2016). These authors used a four-nozzle bioprinter to build an extended tissue of 15 mm × 5 mm × 1 mm (dimensions, respectively, in the *x*, *y*, *z* directions) using cell-laden hydrogels, containing mouse myoblasts, gelatin (Ge), fibrinogen, hyaluronic acid (HA), and glycerol mixed into cell culture medium (Figure 3.13). Furthermore, Pluronic F-127 was used as sacrificial material to create microchannels to mimic vasculature during the maturation of the post-printed construct. Finally, polycaprolactone was employed as supporting material (similar to agarose, discussed earlier). After deposition, fibrinogen was cross-linked with thrombin and all the uncrossed components (HA, Ge, glycerol, Pluronic F-127) were washed out. After 7 days of maturation the construct showed characteristics of well-developed skeletal muscle: The encapsulated myoblasts differentiated and self-organized into unidirectionally organized myotubes with pronounced expression of myosin heavy chain. At this point the construct was implanted into a rat model for structural and

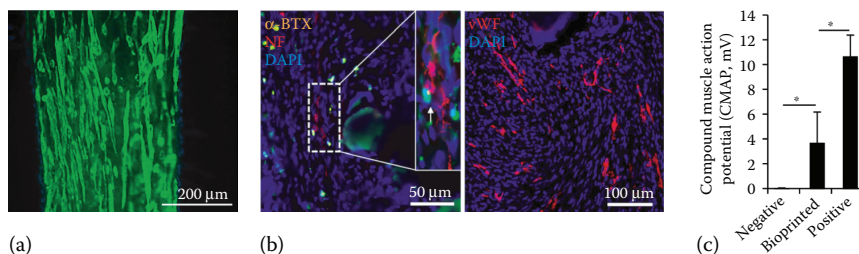


Figure 3.13 Bioprinting skeletal muscle tissue for implantation.

(a) Immunostaining for myosin heavy chain 7 days after differentiation of the myoblasts into myotubes in the printed construct. (b) left: Nerve integration in the implanted muscle evidenced by double staining of neurofilaments (NF+) and α -BTX+ structure (arrow). (b) right: Vascularization of the implanted muscle evidenced by immunostaining for endothelial cells with von Willebrand factor. (c) Functionality of the implanted bioprinted muscle 4 weeks after implantation confirmed by the measurement of the compound muscle potential (as compared to the positive control of the dissected gluteus muscle). (From Kang, H.W. et al., *Nat. Biotechnol.*, 34, 312–319, 2016. With permission.)

functional studies. *In vivo* and *in vitro* (after harvesting) observations showed that the construct maintained the correct fiber structure in the body, became innervated and vascularized, and showed proper function (measured by the compound muscle action potential).

The recent announcement by Organovo (October 5, 2016) about the development of human liver tissue for direct transplantation into patients (<http://...>) is a further indication that therapeutic applications of bioprinting do not belong to science fiction anymore.

3.5 The near future

3.5.1 Toward the elimination of animal trials

In [Section 3.4.2](#), we described the use of bioprinted tissues, in particular liver, in current drug toxicity assays. A further step in pharmaceutical applications can be envisioned by connecting the various bioprinted tissues into a *model organism*, as illustrated in [Figure 3.14](#). Such a structure would be a better model for a drug toxicity assay than the EHLTM discussed earlier.

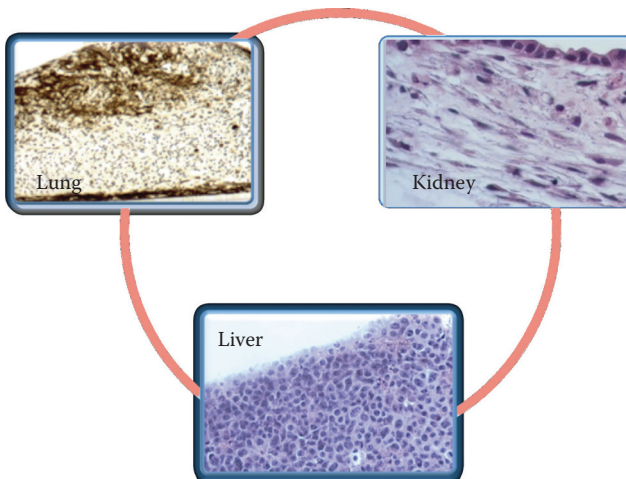


Figure 3.14 Schematic illustration of a model organism. The various bioprinted tissues (such as Organovo's already commercially available Exvive Liver and Kidney Proximal Tubule tissues) could be connected through a common circulatory system (e.g., the bioprinted vascular grafts discussed in [Section 3.3.2](#)) to arrive at a more accurate model for drug toxicity studies.

By combining more tissues it is not unrealistic to assume that such model organisms could soon eliminate the need to use animals in drug development, thus making the process both financially and ethically more acceptable. (Similar attempts are already under way in the organ-on-the-chip community [Oleaga et al., 2016].)

3.5.2 Mitigating the critical shortage of donor organs

With the continuous increase in life expectancy the shortage of donor organs is likely to reach critical proportions. Both the number of donors and the quality of their organs (the latter due to the aging of the population) will be inadequate. Here also, bioprinting could provide a solution in the near future, even if only a temporary one. With progress in the vascularization of the engineered organs, the size of the bioprinted constructs will grow. Thus, as an example, a small liver will soon be possible to build that could keep the patient alive until the right donor is found. Similar effect could be achieved with the relatively thin liver tissues already produced by Organovo. These could be grafted onto the dysfunctional liver (possibly several simultaneously at different locations along the liver) and could temporarily maintain function.

3.5.3 Bioprinting in the operating room

Expeditious reconstruction of damaged organs could be life saving in multiple scenarios, such as a traffic accident, battlefield injury, or burn wound. A bioprinter in the operating room could respond to such demands. The replacement tissue or organ structure could be prepared on the spot using autologous, allogeneic, or even xenogeneic cells if needed and could be immediately implanted into the patient for maturation, given that the body is the ideal bioreactor. Cubo and colleagues have recently used bioprinting to build functional human skin (Cubo et al., 2016). Setting up a similar process in surgical unit could be anticipated. Similarly, scientists at the Wake Forest University Institute of Regenerative Medicine have announced in February 2017 the printing of cells directly onto burn wounds (<http://www.designindaba.com/articles/creative-work/3d-printing-skin-cells-directly-burn-wounds>).

3.6 The more distant realistic future

The ultimate goal of bioprinting is to fabricate fully functional replacement organs. This goal is not likely to be reached in the near future for a number of reasons. Perhaps, the most restrictive one is that such an undertaking would require an astronomical number of live cells: A full-sized human liver, for example, contains an order of 100 billion cells. No technology exists at present to produce these many cells within finite time. Even if such technology is developed, it is unlikely

that a human liver or heart could be reproduced by bioprinting or by any other method that could mimic these human organs—the result of millions of years of evolution and many iterations—in minute details. The human heart is undoubtedly a fantastic machine, the marvel of nature. But is it the best to fulfill its function? It is not impossible that the ongoing progress in engineering in general and bioengineering in particular will lead to a design principle one day for our vital pump that would exceed in its performance of even the organ we carry in our body. What is important to keep in mind is that we do not need to repeat what nature has accomplished. We need to build an organ, imperatively of the patient's own cells that would function at least as well as the one we are born with, if not better.

3.7 Conclusion

In this chapter, we presented a short overview of extrusion bioprinting. As with bioprinting in general, extrusion bioprinting has evolved remarkably since its early days at the start of the century: Printers became more sophisticated (Figure 3.2), the preparation of the bioink got automated and the three phases of pre-processing, processing, and post-processing became more integrated. Extrusion bioprinting has distinguishing features that make its application to tissue and organ engineering particularly suitable. The bioink in this technology can be a minitissue, such as a multicellular spheroid or cylinder, in which cells already reside in a truly 3D environment. With the cylindrical bioink, extrusion can be made continuous. This allows reaching high cell density that is critical for the building of extended living structures. The simultaneous deposition of the cellular and hydrogel components or mixtures of multiple cell types can be conveniently accomplished by using printers with several deposition heads. Damage to cells in the course of printing is minimal.

Applications of extrusion bioprinting, as illustrated here through typical examples, are already broad, encompassing basic research and drug development, with the continuously expanding list. Even though we discussed future applications, both in the near and distant, nobody can predict today with certainty where the technology will eventually lead. With progress in cell culture and bioreactor design, the possibilities are vast. Drug development and testing will continue to benefit from bioprinting and the elimination of the use of animals in preclinical trials is within the horizon. Full therapeutic applications are more distant, but partial remedies to tissue and organ shortage, regeneration, and replacement do not belong to science fiction anymore. The printing of entire solid noble organs in the shape and form identical to that of those in the human body is not likely but, as argued, not necessary.

Bioprinting, as is amply evidenced in this book, is a transformational technology that is fundamentally changing our attitude toward the life sciences and medicine and with all likelihood, will continue to do so.

References

- Albritton, J.L., and J.S. Miller. 2017. 3D bioprinting: Improving in vitro models of metastasis with heterogeneous tumor microenvironments. *Disease Models & Mechanisms*. 10:3–14.
- Ali, M., E. Pages, A. Ducom, A. Fontaine, and F. Guillemot. 2014. Controlling laser-induced jet formation for bioprinting mesenchymal stem cells with high viability and high resolution. *Biofabrication*. 6:045001.
- Arslan-Yildiz, A., R. El Assal, P. Chen, S. Guven, F. Inci, and U. Demirci. 2016. Towards artificial tissue models: Past, present, and future of 3D bioprinting. *Biofabrication*. 8:014103.
- Cohen-Karni, T., B.P. Timko, L.E. Weiss, and C.M. Lieber. 2009. Flexible electrical recording from cells using nanowire transistor arrays. *Proceedings of the National Academy of Sciences of the United States of America*. 106:7309–7313.
- Cubo, N., M. Garcia, J.F. Del Canizo, D. Velasco, and J.L. Jorcano. 2016. 3D bioprinting of functional human skin: Production and in vivo analysis. *Biofabrication*. 9:015006.
- Cui, H., M. Nowicki, J.P. Fisher, and L.G. Zhang. 2017. 3D bioprinting for organ regeneration. *Advanced Healthcare Materials*. 6:1601118.
- Curcio, E., L. De Bartolo, G. Barbieri, M. Rende, L. Giorno, S. Morelli, and E. Drioli. 2005. Diffusive and convective transport through hollow fiber membranes for liver cell culture. *Journal of Biotechnology*. 117:309–321.
- Datta, P., B. Ayan, and I.T. Ozbolat. 2017. Bioprinting for vascular and vascularized tissue biofabrication. *Acta Biomaterialia*. 51:1–20.
- Demirci, U., and G. Montesano. 2007. Single cell epitaxy by acoustic picolitre droplets. *Lab on a Chip*. 7:1139–1145.
- Devillard, R., E. Pages, M.M. Correa, V. Keriquel, M. Remy, J. Kalisky, M. Ali, B. Guillotin, and F. Guillemot. 2014. Cell patterning by laser-assisted bioprinting. *Methods in Cell Biology*. 119:159–174.
- Di Bella, C., A. Fosang, D.M. Donati, G.G. Wallace, and P.F. Choong. 2015. 3D Bioprinting of cartilage for orthopedic surgeons: Reading between the lines. *Frontiers in Surgery*. 2:39.
- Dias, A.D., D.M. Kingsley, and D.T. Corr. 2014. Recent advances in bioprinting and applications for biosensing. *Biosensors*. 4:111–136.
- Duan, B. 2017. State-of-the-art review of 3D bioprinting for cardiovascular tissue engineering. *Annals of Biomedical Engineering*. 45:195–209.
- Ferris, C.J., K.G. Gilmore, G.G. Wallace, and M. In het Panhuis. 2013. Biofabrication: An overview of the approaches used for printing of living cells. *Applied Microbiology and Biotechnology*. 97:4243–4258.
- Forgacs, G., R.A. Foty, Y. Shafir, and M.S. Steinberg. 1998. Viscoelastic properties of living embryonic tissues: A quantitative study. *Biophysical Journal*. 74:2227–2234.
- Forgacs, G., and S. Newman. 2005. *Biological Physics of the Developing Embryo*. Cambridge University Press, Cambridge.
- Foty, R. 2011. A simple hanging drop cell culture protocol for generation of 3D spheroids. *Journal of Visualized Experiments: Jove*. 51:e2720.
- Foty, R.A., C.M. Pflieger, G. Forgacs, and M.S. Steinberg. 1996. Surface tensions of embryonic tissues predict their mutual envelopment behavior. *Development*. 122:1611–1620.
- Foty, R.A., and M.S. Steinberg. 2005. The differential adhesion hypothesis: A direct evaluation. *Developmental Biology*. 278:255–263.
- Gudapati, H., M. Dey, and I. Ozbolat. 2016. A comprehensive review on droplet-based bioprinting: Past, present and future. *Biomaterials*. 102:20–42.
- Guillemot, F., A. Souquet, S. Catros, and B. Guillotin. 2010a. Laser-assisted cell printing: Principle, physical parameters versus cell fate and perspectives in tissue engineering. *Nanomedicine*. 5:507–515.

- Guillemot, F., A. Souquet, S. Catros, B. Guillotin, J. Lopez, M. Faucon, B. Pippenger, R. Bareille, M. Remy, S. Bellance, P. Chabassier, J.C. Fricain, and J. Amedee. 2010b. High-throughput laser printing of cells and biomaterials for tissue engineering. *Acta Biomaterialia*. 6:2494–2500.
- Guillotin, B., A. Souquet, S. Catros, M. Duocastella, B. Pippenger, S. Bellance, R. Bareille, M. Remy, L. Bordenave, J. Amedee, and F. Guillemot. 2010. Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials*. 31:7250–7256.
- Hamilton, G. 1998. Multicellular spheroids as an in vitro tumor model. *Cancer Letters*. 131:29–34.
- Hoch, E., G.E. Tovar, and K. Borchers. 2014. Bioprinting of artificial blood vessels: Current approaches towards a demanding goal. *European Journal of Cardio-Thoracic Surgery* 46:767–778.
- Irvine, S.A., and S.S. Venkatraman. 2016. Bioprinting and differentiation of stem cells. *Molecules*. 21:1188.
- Itoh, M., K. Nakayama, R. Noguchi, K. Kamohara, K. Furukawa, K. Uchihashi, S. Toda, J. Oyama, K. Node, and S. Morita. 2015. Scaffold-free tubular tissues created by a bio-3D printer undergo remodeling and endothelialization when implanted in rat aortae. *PloS One*. 10:e0136681.
- Jakab, K., A. Neagu, V. Mironov, R.R. Markwald, and G. Forgacs. 2004. Engineering biological structures of prescribed shaped using self-assembling multicellular systems. *Proceedings National Academy of Sciences of the United States of America*. 101:2864–2869.
- Jakab, K., C. Norotte, B. Damon, F. Marga, A. Neagu, C.L. Besch-Williford, A. Kachurin et al. 2008. Tissue engineering by self-assembly of cells printed into topologically defined structures. *Tissue Engineering Part A*. 14:413–421.
- Jakab, K., C. Norotte, F. Marga, K. Murphy, G. Vunjak-Novakovic, and G. Forgacs. 2010. Tissue engineering by self-assembly and bio-printing of living cells. *Biofabrication*. 2:022001.
- Jana, S., and A. Lerman. 2015. Bioprinting a cardiac valve. *Biotechnology Advances*. 33:1503–1521.
- Justice, B.A., N.A. Badr, and R.A. Felder. 2009. 3D cell culture opens new dimensions in cell-based assays. *Drug Discovery Today*. 14:102–107.
- Kang, H.W., S.J. Lee, I.K. Ko, C. Kengla, J.J. Yoo, and A. Atala. 2016. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology*. 34:312–319.
- King, S.M., J.W. Higgins, C.R. Nino, T.R. Smith, E.H. Paffenroth, C.E. Fairbairn, A. Docuayan et al. 2017. 3D proximal tubule tissues recapitulate key aspects of renal physiology to enable nephrotoxicity testing. *Frontiers in Physiology*. 8:123.
- Knight, E., and S. Przyborski. 2015. Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. *Journal of Anatomy*. 227:746–756.
- Knowlton, S., S. Onal, C.H. Yu, J.J. Zhao, and S. Tasoglu. 2015. Bioprinting for cancer research. *Trends in Biotechnology*. 33:504–513.
- Kolesky, D.B., K.A. Homan, M.A. Skylar-Scott, and J.A. Lewis. 2016. Three-dimensional bioprinting of thick vascularized tissues. *Proc. Natl. Acad. Sci. USA*. 113:3179–3184.
- Kolesky, D.B., R.L. Truby, A.S. Gladman, T.A. Busbee, K.A. Homan, and J.A. Lewis. 2014. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials*. 26:3124–3130.
- Kosztin, I., G. Vunjak-Novakovic, and G. Forgacs. 2012. Colloquium: Modeling the dynamics of multicellular systems: Application to tissue engineering. *Reviews of Modern Physics*. 84:1791–1805.

- Kunz-Schughart, L.A., M. Kreutz, and R. Knuechel. 1998. Multicellular spheroids: A three-dimensional in vitro culture system to study tumour biology. *International Journal of Experimental Pathology*. 79:1–23.
- Langer, R., and J.P. Vacanti. 1993. Tissue engineering. *Science*. 260:920–926.
- Li, J., M. Chen, X. Fan, and H. Zhou. 2016. Recent advances in bioprinting techniques: Approaches, applications and future prospects. *Journal of Translational Medicine*. 14:271.
- Lin, R.Z., and H.Y. Chang. 2008. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal*. 3:1172–1184.
- Marga, F., K. Jakab, C. Khatiwala, B. Shephard, S. Dorfman, B. Hubbard, S. Colbert, and F. Gabor. 2012. Toward engineering functional organ modules by additive manufacturing. *Biofabrication*. 4:022001.
- Mironov, V., R.P. Visconti, V. Kasyanov, G. Forgacs, C.J. Drake, and R.R. Markwald. 2009. Organ printing: Tissue spheroids as building blocks. *Biomaterials*. 30:2164–2174.
- Napolitano, A.P., P. Chai, D.M. Dean, and J.R. Morgan. 2007. Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels. *Tissue Engineering*. 13:2087–2094.
- Neagu, A., K. Jakab, R. Jamison, and G. Forgacs. 2005. Role of physical mechanisms in biological self-organization. *Physical Review Letters*. 95:178104.
- Ng, W.L., J.M. Lee, W.Y. Yeong, and M. Win Naing. 2017. Microvalve-based bioprinting—process, bio-inks and applications. *Biomaterials Science*. 5:632–647.
- Ng, W.L., S. Wang, W.Y. Yeong, and M.W. Naing. 2016. Skin bioprinting: Impending reality or fantasy? *Trends in Biotechnology*. 34:689–699.
- Nguyen, D.G., J. Funk, J.B. Robbins, C. Crogan-Grundy, S.C. Presnell, T. Singer, and A.B. Roth. 2016. Bioprinted 3D primary liver tissues allow assessment of organ-level response to clinical drug induced toxicity in vitro. *PLoS One*. 11:e0158674.
- Norotte, C., F.S. Marga, L.E. Niklason, and G. Forgacs. 2009. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*. 30:5910–5917.
- Oleaga, C., C. Bernabini, A.S. Smith, B. Srinivasan, M. Jackson, W. McLamb, V. Platt et al. 2016. Multi-organ toxicity demonstration in a functional human in vitro system composed of four organs. *Scientific Reports*. 6:20030.
- Owens, C.M., F. Marga, G. Forgacs, and C.M. Heesch. 2013. Biofabrication and testing of a fully cellular nerve graft. *Biofabrication*. 5:045007.
- Ozbolat, I.T., and M. Hospodiuk. 2016. Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials*. 76:321–343.
- Ozbolat, I.T., W. Peng, and V. Ozbolat. 2016. Application areas of 3D bioprinting. *Drug Discovery Today*. 21:1257–1271.
- Pati, F., J. Gantelius, and H.A. Svahn. 2016. 3D bioprinting of tissue/organ models. *Angewandte Chemie*. 55:4650–4665.
- Perez-Pomares, J.M., and R.A. Foty. 2006. Tissue fusion and cell sorting in embryonic development and disease: Biomedical implications. *BioEssays*. 28:809–821.
- Phillips, H.M., and M.S. Steinberg. 1978. Embryonic tissues as elasticoviscous liquids. I. Rapid and slow shape changes in centrifuged cell aggregates. *Journal of Cell Science*. 30:1–20.
- Ravi, M., V. Paramesh, S.R. Kaviya, E. Anuradha, and F.D. Solomon. 2015. 3D cell culture systems: Advantages and applications. *Journal of Cellular Physiology*. 230:16–26.
- Ringeisen, B.R., H. Kim, J.A. Barron, D.B. Krizman, D.B. Chrisey, S. Jackman, R.Y. Auyeung, and B.J. Spargo. 2004. Laser printing of pluripotent embryonal carcinoma cells. *Tissue Engineering*. 10:483–491.
- Seol, Y.J., H.W. Kang, S.J. Lee, A. Atala, and J.J. Yoo. 2014. Bioprinting technology and its applications. *European Journal of Cardio-Thoracic Surgery*. 46:342–348.

- Shafiee, A., M. McCune, G. Forgacs, and I. Kosztin. 2015. Post-deposition bioink self-assembly: A quantitative study. *Biofabrication*. 7:045005.
- Skardal, A., and A. Atala. 2015. Biomaterials for integration with 3-D bioprinting. *Annals of Biomedical Engineering*. 43:730–746.
- Steinberg, M.S. 2007. Differential adhesion in morphogenesis: A modern view. *Current Opinion Genetics & Development*. 17:281–286.
- Tasoglu, S., and U. Demirci. 2013. Bioprinting for stem cell research. *Trends in Biotechnology*. 31:10–19.
- Tian, B., J. Liu, T. Dvir, L. Jin, J.H. Tsui, Q. Qing, Z. Suo, R. Langer, D.S. Kohane, and C.M. Lieber. 2012. Macroporous nanowire nanoelectronic scaffolds for synthetic tissues. *Nature Materials*. 11:986–994.
- van Duinen, V., S.J. Trietsch, J. Joore, P. Vulto, and T. Hankemeier. 2015. Microfluidic 3D cell culture: From tools to tissue models. *Current Opinion in Biotechnology*. 35:118–126.
- Vijayavenkataraman, S., W.F. Lu, and J.Y. Fuh. 2016. 3D bioprinting of skin: A state-of-the-art review on modelling, materials, and processes. *Biofabrication*. 8:032001.
- Visscher, D.O., E. Farre-Guasch, M.N. Helder, S. Gibbs, T. Forouzanfar, P.P. van Zuijlen, and J. Wolff. 2016. Advances in bioprinting technologies for craniofacial reconstruction. *Trends in Biotechnology*. 34:700–710.
- Whitesides, G.M., and B. Grzybowski. 2002. Self-assembly at all scales. *Science*. 295:2418–2421.
- Wilson, W.C., Jr., and T. Boland. 2003. Cell and organ printing I: Protein and cell printers. *The Anatomical Record* 272:491–496.
- Zhang, Y.S., M. Duchamp, R. Oklu, L.W. Ellisen, R. Langer, and A. Khademhosseini. 2016. Bioprinting the cancer microenvironment. *ACS Biomaterials Science & Engineering*. 2:1710–1721.
- Zhao, Y., R. Yao, L. Ouyang, H. Ding, T. Zhang, K. Zhang, S. Cheng, and W. Sun. 2014. Three-dimensional printing of Hela cells for cervical tumor model in vitro. *Biofabrication*. 6:035001.



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Chapter 4 Laser-based 3D bioprinting

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4.1 Introduction

Three-dimensional (3D) printing has been used for industrial rapid prototyping for decades. These techniques have become increasingly utilizable for tissue engineering (TE) applications. Today, the primary goal in TE is to fabricate functional tissue and organ substitutes through 3D bioprinting for implantation *in vivo* or for improved *in vitro* models (Figure 4.1) [1–3]. Traditional TE methodology is applied top–down, through assembly of cells and materials, via seeding of

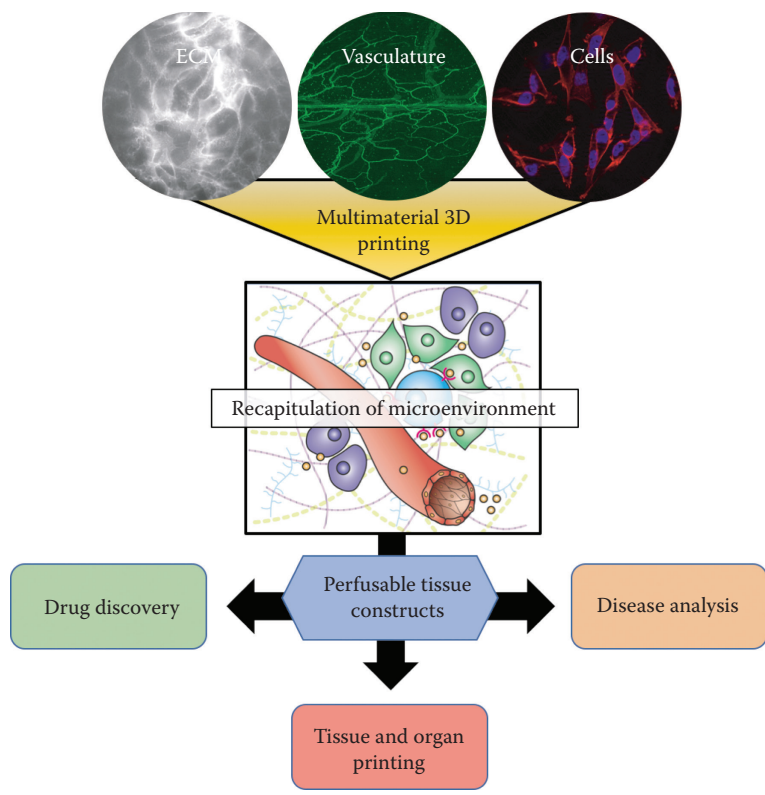


Figure 4.1 3D bioprinting overview.

biodegradable, porous, and prefabricated scaffolds. Preclinical experiments and the first clinical implantations of scaffold-based TE constructs [4] have revealed that one of the prevailing problems of the top-down approach to engineering 3D-tissues is the poor penetration of viable cells inside the scaffold, made worse by increasing the scaffold thickness. This is due to limited transport of nutrients and waste inside the porous materials [5]. These experiments, and many others, have highlighted two fundamental obstacles in TE: (1) the histological complexity of tissue microenvironments and (2) the inability to maintain large masses of living cells on transfer from *in vitro* culture conditions into the host *in vivo*.

In vivo, most cells do not survive more than a few hundred micrometers from the nearest capillary, due to diffusion limitations [6–8]. Likewise, *in vitro*, without an offsetting transport technique such as flow reactors [9], or oxygen releasing

polymers [10], cell survival is limited to the outer few hundred micrometers of a tissue construct [11,12]. The distance between adjacent cell groups in a multicellular construct, as well as the arrangement of distinct cell populations within a pattern permutation may influence the mode and intensity of cell–cell communication, thereby affecting the context of cell behavior and genetic expression. Thus, next-generation biofabrication of spatially defined constructs requires precise control in the microscale positioning of homogeneous and/or heterogeneous cell groups.

Knowledge of developmental biology can provide valuable insights into TE [13]. For example, during embryonic development, tissues and organs are formed in the absence of solid scaffolds. Furthermore, although overall embryonic development is a relatively slow process, certain essential steps during histogenesis and organogenesis are relatively fast. A primary imperative in TE is a rapid tissue biofabrication [14]. As such, an alternative method for the assembly of cells and biomaterials that seeks to address the two critical TE hurdles and is defined by a developmental biology-inspired, bottom–up approach that relies on the assembly of building blocks, mimicking native functional units, into larger tissue constructs [15,16].

Most important, a challenge that this and all other approaches to TE are facing is the balanced combination of powerful biological insight into technological imperatives and constraints. Meaning that, even if it is possible to recapitulate or mimic certain essential developmental biology processes in TE technologies, to develop commercially sound technologies, the bioassembly and maturation processes must be both accelerated and automated.

Direct-write techniques, adapted from technologies designed to print plastics and metals [17], epitomize a bottom–up approach and are being used with increasing frequency to deposit biomaterials on diverse types of substrates in TE. The *direct-write* paradigm refers to the deliberate placement of cells, biologics, or biomimetic material subunits into desired pattern positions [18]. In general, direct-write processes rely on the dispensing, transfer, or printing of discrete voxels (volume–pixels) at computer-controlled locations on a substrate [19]. These voxels serve as the building blocks for the lines (1D), sheets or layers (2D), and macroscopic 3D-structures required to fabricate the scaffolds, extracellular matrix (ECM), and cellular constructs.

Direct-write technologies integrate computer-aided design/computer-aided manufacturing (CAD/CAM) for high-level precision in spatial positioning of voxels of bioink within a co-fabricated 3D-hydrogel matrix [20]. Three main direct-write techniques are generally applied to deposit voxels from the bottom up: (1) inkjet printing [21], (2) extrusion-based printing (also called bioplotting,

syringe-based printing, and robotic dispensing) [22], and (3) laser-based printing (also known as laser bioprinting, laser-induced forward transfer [LIFT], laser-assisted bioprinting [LAB], biological laser-printing [BioLP], and matrix-assisted pulsed laser evaporation direct-write [MAPLE-DW]) [23].

By taking advantage of built-in printer CAD and inherent microdispensing of *inks* (or bioinks), inkjet bioprinters [21] have been utilized to fabricate tissue-engineered constructs. The printing mechanism is noncontact and dispenses dropwise suspensions onto target surfaces; very small volumes can be generated at high repetition rates. However, only materials with low cell densities and low viscosity can be printed due to the shear forces experienced as the solution passes through the nozzle [24]. In addition, there is a lower limit to the number of cells deposited (low printing resolution) and only one bioink can be deposited with one print head. The low resolution results because cells per drop are only controlled by the overall cell density of the suspensions.

Nozzles are also intrinsic in extrusion bioprinting techniques [22]. The biomaterial is pressed (extruded) through them as a strand. Typical nozzle inner diameters range between 50 and 1000 μm . The use of smaller nozzles results in high shear forces like those seen in inkjet printing, and thus, the same limitations apply; only materials with low viscosity and low cell density can be printed and there is no *a priori* prediction of the cell density for a single cell type. Because of this, larger nozzles with diameters between 500 and 1000 μm are used more often and the printing resolution is relatively low. The lower resolutions ($\sim 500 \mu\text{m}$) seen in extrusion printing are inadequate to fabricate complex tissues since in *in vivo*, the distance between neighboring blood vessels can be as small as $130 \pm 60 \mu\text{m}$ [25].

Laser-based bioprinting has the advantage of printing materials with high cell densities and high viscosity at high resolutions. As an optical technique, it is possible to visually identify and position cells and biomaterials in real time for subsequent deposition. Thus, laser direct-write (LDW) techniques provide an attractive alternative for bioprinting multicellular constructs in spatially ordered patterns at a near single cell resolution. It is a noncontact, orifice-free group of techniques that offers the ability to deposit biological materials with microscale precision ($\pm 5 \mu\text{m}$) [18,26] and deposit objects over two orders-of-magnitude in voxel size (up to 600 μm) via expansion of the laser cross-sectional area [27]. This is important as the complexity of natural tissue varies on the micrometer scale. The biologic heterogeneity of laser direct-written tissue is $\sim 10\text{--}20 \mu\text{m}$ (approximate size of a single cell) in three dimensions (x, y, z) or 1,300 dots per inch (dpi). More simply, the voxel cubes can be varied with perfect fidelity on this order. The limitations in this approach are more in the kinematic movements of stages. The importance of this metric cannot be understated in terms of the precision and time required to convert

a macroscopic input source of various biologic materials from components to functional autologous tissue. Thus, CAD/CAM laser direct-write (LDW) 3D printing offers a powerful tool for fabricating microscale structures for TE and regenerative medicine applications.

This chapter begins with an overview of laser-based techniques before moving on to discuss how laser-based 3D printing addresses core issues that are faced in TE. In particular, how a technique generally seen as a precise 2D-patterning technique can be utilized to fabricate 3D constructs en route to tissue and organ printing for both clinical and investigative purposes.

4.2 Laser printing overview

LDW printing was first used to deposit patterns of materials for fabrication of superconducting thin-films [28] and electronic circuit elements. [17,29,30] Thick films of metals and composites have been printed to construct conductors, capacitors, and resistors, respectively, with a spatial resolution between 1–3 μm using laser deposition techniques [17]. It was this resolution and reproducibility that made LDW techniques desirable for adaptation to biomedical applications [31].

A typical laser-based printing system is generally composed of four basic elements: (1) a pulsed laser source, (2) beam delivery optics, (3) a target (the ribbon) coated with the material to be printed, and (4) a receiving substrate opposite to the coated print ribbon. The laser transparent print ribbon is a multilayered component comprising the ribbon support, which is transparent to the laser radiation wavelength and typically UV-grade quartz, an energy-absorbing layer that differs on the basis of technique, and a transfer layer (bioink) composed of the biological material to be printed. The bioink coating is generally composed of a layer of biomaterials that are adhered to a biological polymer through initial cellular attachment or uniformly suspended in a thin layer of liquid (usually cell culture medium mixed with glycerol) or a hydrogel. The receiving substrate is likewise coated with a biopolymer or cell culture medium to promote and maintain cellular adhesion and sustain growth. The receiving substrate is mounted on motorized stages and positioned facing the cell-coated side of the ribbon.

To deposit material, a pulsed laser beam is transmitted through the ribbon and is used to propel cells from the ribbon to the receiving substrate. The rapid volatilization and subsequent expansion of the cellular support layer on the ribbon create the force that is necessary to allow the cells to cross the small (700–2000 μm [32]) gap between the ribbon and the receiving substrate (Figure 4.2).

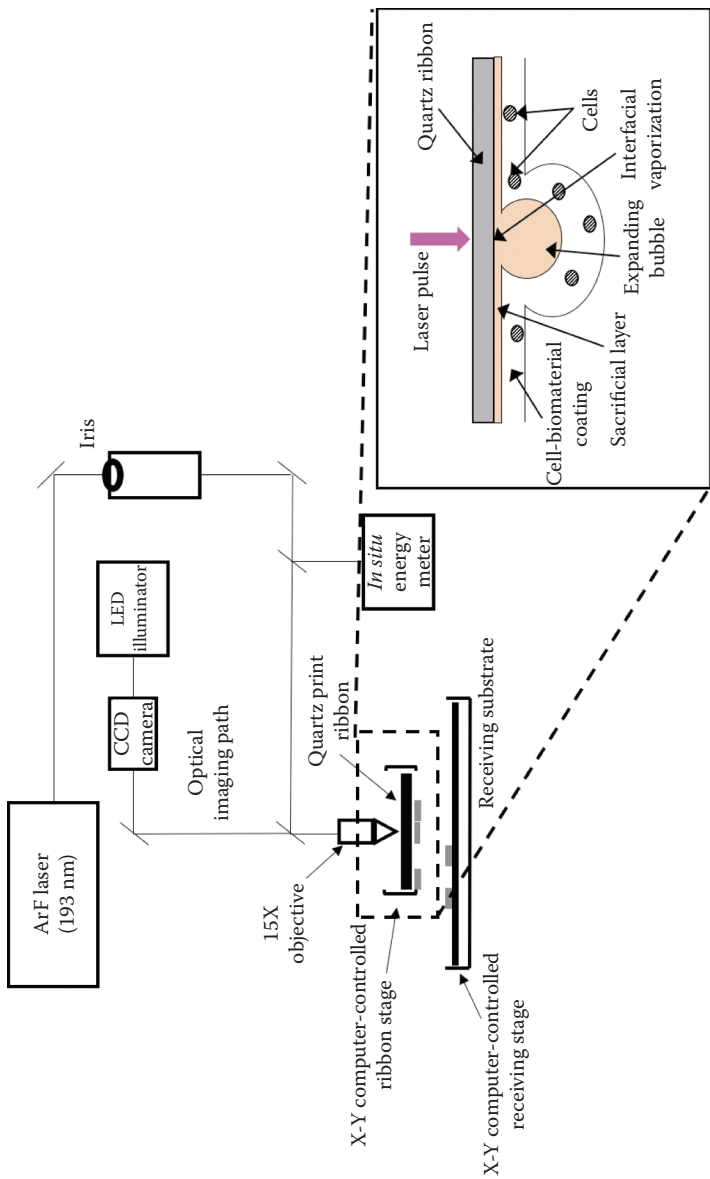


Figure 4.2 Schematic of an example laser-based 3D printing setup. Laser-based 3D printing schematic with an *in situ* camera enables real-time targeting of biomaterials for printing. Through CAD/CAM control, biomaterials are additively printed into spatially defined patterns. Laser deposition mechanism is shown in the magnified cutout; a single pulse of 193 nm excimer laser causes the formation of a local vapor pocket that ejects a desired amount of biomaterial.

4.3 Laser-based techniques

Laser-based printing techniques share similarities in methodology for the direct writing. The multiple different techniques are generally derived or modified versions of one of three techniques: (1) LIFT, (2) absorbing film-assisted laser-induced forward transfer (AFA-LIFT), and (3) MAPLE-DW.

4.3.1 LIFT

LIFT [28,33–37] utilizes high-power density, typically femtosecond, laser pulses to deposit biomaterials from a source onto a receiving substrate. In this process, a laser transparent quartz disk or *ribbon* is coated with a thin film of metal or another laser-absorbing biocompatible material to protect from the potentially damaging effects of the high-power laser pulses. Biomaterials are suspended on the underside of the ribbon. The laser pulse heats the interface of the laser-absorbing thin film and the source substrate, resulting in a melt front that propagates through the film to the free surface. At the free surface, the biocompatible material at the quartz/suspension interface is superheated past its boiling point and the subsequent vapor-induced pressure propels biomaterial forward toward the receiving substrate [28,38].

4.3.2 AFA-LIFT

AFA-LIFT [39–41] is a similar technique to LIFT; however, it utilizes a thick (~100 nm) sacrificial layer of a metal on the ribbon to interact with the laser. Various metal layers, such as Au, Ag, Ti, and TiO₂, have been used with success. A version of AFA-LIFT, called BioLP [42–45], makes use of motorized receiving stages and a coordinated camera to help in focusing the laser. The technique uses a sacrificial metal layer (75–100 nm thick) to have a rapid thermal expansion. This produces a pressure wave that propels small volumes from the biomaterial-laden ribbon to the receiving substrate to minimize biomaterial heating as demonstrated by high-speed imaging showing a stream of fluid, instead of vapor leaving the ribbon [46]. Biomaterials, such as cells, have been suspended in both culture medium and various hydrogels.

4.3.3 MAPLE-DW

MAPLE-DW [32,43,47] is schematically similar to AFA-LIFT and operates on similar principles to those of LIFT with the exception that MAPLE-DW utilizes a low-powered, pulsed laser operating in the UV or near-UV region (Figure 4.2) [40,48,49]. The low-fluence laser requires a volatile matrix (or sacrificial layer), such as gelatin, to assist in the ejection of the material of interest. The underside of the ribbon is coated with the biomaterials, such as cells,

to be transferred. The material transfer occurs following vaporization of the matrix at the ribbon—matrix interface. Localized vaporization of the sacrificial matrix generates a sufficient plume for the transfer spot to be propelled and fall onto a receiving substrate under the influence of kinetic energy and its own weight. The low power and short wavelength (UV) help to ensure that the laser does not penetrate to the biomaterial attachment layer. Initial biomaterial/cell attachment to the transparent biopolymer layer is required on the ribbon. As MAPLE-DW is coupled with an area camera and computer-controlled x , y , z translation stages, real-time visualization allows for precise targeting on the ribbon and achieves single-cell resolution. MAPLE-DW provides CAD/CAM control and processing along with selective voxel patterning for the creation of 3D constructs.

4.4 Turning the precise 2D/2.5D spatial resolution of LDW into 3D tissues

Higher resolution processes, such as laser-based approaches, inevitably print fewer cells and less biomaterial per droplet than inkjet or extrusion printing techniques [50]. Although laser printing has been implemented for patterning and printing using various biomaterials as well as cellular bioinks, they are generally considered as 2D-printing processes [18,51]. Some interesting 2D constructs include 2D branch and stem structures made from human umbilical vein endothelial cells (HUVECs) and human umbilical vein smooth muscle cells to investigate the interaction of these two cells [52] and 2D chess board-like cardiac patch comprising HUVECs and human mesenchymal stem cells for cardiac regeneration [53]. Ultimately, 3D constructs are of paramount interest and fewer studies have been reported regarding the fabrication of complex and spatially heterogeneous 3D patterns. However, the laser-based techniques to fabricate 3D structures are continuously being iterated on. Current strategies for laser-based 3D bioprinting include layer-by-layer hydrogel stacking, microscale encapsulation, spheroid printing, and free-form fabrication.

4.4.1 Layer-by-layer hydrogel stacking

As the high-resolution laser-based techniques print less material per droplet, it can be impractical to print both cells and scaffolding together, which leads to the reputation of laser techniques as 2D tools. The simplest tactic to extend the laser-based printing techniques into 3D has been to serially print cells alone (in media) onto successive layers of hydrogel in an additive fashion. This approach is epitomized by the work of Barron et al. [42] when they printed three patterns of osteosarcoma cells to three successive 100 μm MatrigelTM hydrogel layers.

However, in this example, using successive hydrogel layering to fabricate 3D structures will provide insufficient mechanical support that will ultimately limit the size of the construct and may cause creeping of printed patterns [50,54]. In another example, a natural hydrogel consisting of a fibrin precursor and hyaluronic acid served as the cell carrier and environmental material [23]. The use of a stiffer hydrogel material allowed stacking to a greater degree. Continuing along this trend Pirlo et al. [50] introduced Biopapers. Biopapers are thin polymer/hydrogel 3D-scaffold sheets that possess more rigidity and act as substrates for cell and biomaterial printing. Stacking these rigid scaffold sheets provides a solution to the mechanical support issues seen with layering of hydrogels. Biopapers are patterned with high-resolution laser-based printing techniques and after stacking they would enable thick 3D-tissue constructs to be created with well-defined 3D-cell arrangements. Layer-stacking approaches allow mimicking the cell–cell arrangements of native tissue types and could be used in prevascularization of engineered thick tissues.

4.4.2 Microscale encapsulation

Encapsulation and printing using hydrogels are oft-employed techniques in TE [55,56]. Conventional bulk hydrogel encapsulation suffers from limited nutrient and waste exchange [57], and poor spatial control of hydrogel constituents [56]. Unfavorable hydrogel diffusion kinetics can lead to necrosis of encapsulated cells, once the thickness exceeds the diffusion limit of the hydrogel [58]. Techniques to fabricate microspheres (which includes microbeads and microcapsules) enable the engineering of artificial microenvironments that resemble *in vivo* niches and seek to address these limitations. Several research groups have married these microscale encapsulation techniques to bottom–up TE approaches (Figure 4.3). This combination allows for the fabrication and deposition of cell-laden, physiologically relevant hydrogel subunits that serve as building blocks to create more complicated heterogeneous constructs [59].

The precise placement of these subunits into spatially ordered patterns has broad implications for biomaterial processing and biomedical applications. Patterning cell-laden microspheres have proven useful as cell-based combinatorial arrays for lab-on-a-chip devices [60] and building blocks for engineered tissue construction [37,59,61]. Microscale patterning technologies facilitate the placement of solid microspheres or engineered hydrogel microenvironments into specific positions on substrate areas. The ability to selectively deposit microspheres on a sphere-by-sphere basis allows researchers the freedom to fabricate custom patterns, with the benefits of a bottom–up TE approach.

Both solid and hydrogel microspheres are attractive subunits for bottom–up engineering because of their well-defined shape, tunable mechanical properties, definable surface chemistry, and biomimetic 3D-microenvironment. In addition,

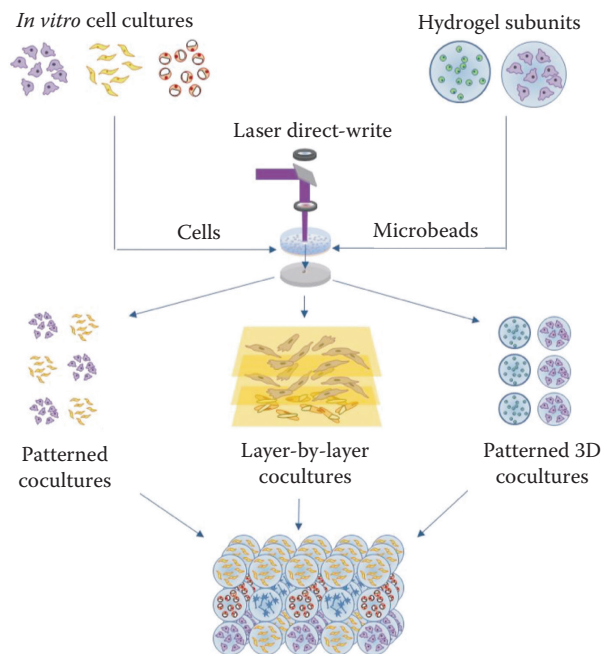


Figure 4.3 Schematic overview of bottom-up fabrication by laser-based 3D bioprinting.

biochemical properties (e.g., composition, presentation of ligands or receptors, and encapsulated cell density) can be customized without affecting the overall printing processes. The ability to localize functional or bioactive subunits into a variety of configurations broadens the applicability of microsphere patterns for biomedical devices and engineered tissue constructs. Particle selection and positioning according to their physical size defines the sensitivity and resolution of actuation in many of the applications. Thus, bottom-up printing techniques are ideal for size-selective positioning of microsphere subunits into spatially ordered patterns.

Hydrogel microcapsules are spherical microstructures, consisting of a semi-permeable shell surrounding an aqueous core [62,63]. In contrast to solid microbeads, cells encapsulated in microcapsules grow and proliferate on the interior shell surface [64], and must produce matrix and/or self-assemble into aggregates to grow in 3D [65]. Typically, microcapsules are fabricated through the processing of microbeads, by coating charged hydrogel microbeads with a polymer of opposite charge (e.g., a negatively charged alginate microbead and positively charged poly-L-lysine [PLL] or chitosan) [66]. This process forms

a shell in a phenomenon known as polyelectrolyte complexation [67,68]. The interior of the bead can then be solubilized by using a reverse cross-linking agent [68]. The resulting structure is a shell retaining the microbead payload in an aqueous core.

4.4.3 Spheroids

A natural extension of printing microenvironments in microcapsules would be to simply print multicellular spheroids or aggregates (microtissues, tissue spheroids) using laser deposition. Multicellular spheroids or organoids aggregated from cells *in vitro* have been exploited widely as convenient 3D-model systems to mimic normal or pathological tissues for biomedical and tumor research for several decades [14,69]. Continued work has also indicated that spheroid culture can preserve biological properties of freshly isolated or stem cell-derived cells [70,71]. As such, they constitute unique, living building blocks to form complex tissue architectures through tissue fusion, the process by which two or more multicellular spheroids fuse to form one larger structure; an important process in some strategies for layer-by-layer biofabrication [71–74].

As the prevalence of spheroid-based 3D-cell culture gains more traction in biomedical research, the need for a suitable tool to manipulate spheroids individually or in parallel also increases [69]. Like individual cells, LDW techniques can be used to print cellular aggregates such as spheroids (Figure 4.4) [75]. Precise placement of multicellular spheroid subunits as building blocks into spatially ordered patterns allows for the creation of more complicated heterogeneous constructs with microscale precision ($\pm 5 \mu\text{m}$) [59,63]. In addition, spheroids such as embryoid bodies can even be fabricated using LDW printing. Dias et al. showed that by varying printing parameters, they could control the local cell density and colony size independently; at prescribed spatial locations they could control the size and location of the resulting fabricated embryoid body [76].

4.4.4 Free-form constructs

To reiterate, one of the fundamental goals in the scientific and engineering community is to achieve the on-demand fabrication of 3D-human tissue/organ structures [14,51,77]. The complexity and sophistication of constructs have gradually increased with the development and growth of printing techniques. Laser-based 3D printing is no different. Laser printing began as a 2D-technique offering high resolution with precise spatial control. The technique continues to evolve and mature into a 3D-fabrication technique. For example, as discussed earlier, multilayer hydrogel-cell planar structures have been fabricated by laser printing cells or cell aggregates as individual droplets onto a hydrogel layer and then covered with subsequent hydrogel

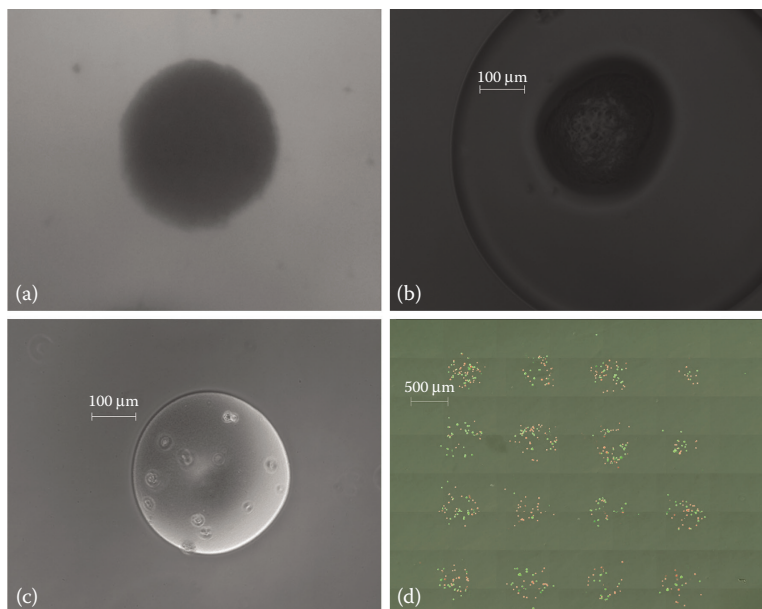


Figure 4.4 Example of laser-based printing images. (a) BJ skin fibroblast (ATCC CRL-2522)-cell spheroid visualized on the quartz ribbon prior to laser deposition. (b) BJ skin fibroblast (ATCC CRL-2522)-cell spheroid imaged with an inverted microscope (AxioObserver Z1, Carl Zeiss, Thornwood, NY) after laser-based printing. While not the same spheroid as pictured in [Figure 4.4\(a\)](#), they are the same size. Laser software does not allow addition of scale bar annotation. (c) Microsphere containing MDA-MB-231 cells post-print. (d) 4×4 array of clusters of cocultured MDA-MB-231 cells (red) and MCF-7 cells (green) generated by LDW.

layers to build 3D constructs [23,42,50]. However, with these stackable approaches, multiple steps are required to add onto each layer and ultimately fabricate a 3D construct. A less time-consuming alternative would be free-form fabrication. This approach was demonstrated first by Yan et al. [78] who utilized a laser-based printing technique to fabricate alginate long tubes and annular constructs. Moreover, these tubular constructs are particularly noteworthy as they are representative of vascular-like structures. Nearly every path to the development of substitute organs necessitates addressing the diffusion limitations inherent in 3D-printed tissue constructs due to the lack of a nutrient/waste handling vasculature. In fact, the likely first step toward successful organ printing is to fabricate various complex 3D-constructs/structures, especially 3D-tubular structures. For thick tissues and organs, vascularization is crucial for the regulation of nutrients, cell function, and ultimately survival. As such, laser printing of 3D-tubular constructs can be considered as an important step toward successful organ printing.

In subsequent work, alginate tubes were printed in the form of both straight and Y-shaped, bifurcated tubes using laser fabrication [79]. This demonstrated that 3D-cellular tubes, including constructs with bifurcated overhang structures, could be fabricated under optimal printing conditions using laser printing. The printing of overhang and spanning structures was achieved using a dual-purpose cross-linking solution, which also functioned as a support material [80]. In addition, fibroblast-containing alginate tubes have been successfully printed in a layer-by-layer fashion using laser deposition with immediate post-printing cell viabilities, as well as after 24-h incubation, above 60% for printed straight and Y-shaped fibroblast containing alginate tubes [80]. Although the viability is lower than other printing techniques such as inkjet [81] and other applications of laser printing [56,82], free-form printing that maintains microscale precision represents an exciting path forward for tissue and organ laser printing.

4.5 Applications

Laser-based 3D bioprinting has the ability to create intricate user-generated constructs for a variety of tissues. The control of cell and biomaterial positioning can be utilized to create spatially precise biological constructs of cells, spheroids, and/or microspheres in a bottom-up, voxel-by-voxel fashion. The application of laser 3D printing can therefore have a significant impact on applications in many research areas, including TE and regenerative medicine. Demonstrations of the 3D potential of laser-based 3D-printing techniques are summarized in the following sections.

4.5.1 Vasculature

Native vasculature consists of cellularly heterogeneous tubular structures, typically having multiple bifurcated/branching features in various orientations that extend throughout a tissue. Thus, the fabrication of such bifurcated tubular structures has been the focus of numerous bioprinting studies, and the construction of 3D-heterogeneous vasculature has widely been recognized as a key challenge in the progression of organ printing [78,83–89].

Classical approaches to vascularization focus on the stimulation of vascular ingrowth into a tissue construct [90]. This is done through material property optimization and incorporation of growth factors. This angiogenic route must contend with a slow average growth rate of newly developing microvessels ($\sim 5 \mu\text{m/h}$) [91]. The larger the implant the more exacerbated the issue and therefore is associated with tissue loss due to hypoxic conditions.

The concept of *prevascularization* has gained momentum as an alternative to classical vascularization approaches in TE. Prevascularization focuses

on the generation of preformed microvascular networks as part of the tissue construct itself or rather as part of the bottom-up process. After implantation, these fabricated networks can be more rapidly perfused with blood from the surrounding host microvasculature or by surgical anastomosis of blood vessels [92,93]. Prevascularization fits into the laser-based 3D-printing paradigm due to its inherent position with the bottom-up approach to TE and the high-resolution ability of laser-based printing techniques. Approaches by Xiong et al. and Kingsley et al. demonstrate the effectiveness of laser 3D printing to fabricate tubular structures at the microscale [63,79].

4.5.2 Skin

Skin is simple relative to other tissues and is often one of the first tissues researchers attempt to replicate when utilizing 3D printing techniques. In addition, as an applied tissue model, skin has a layered configuration (dermis and epidermis), making it an ideal candidate for laser-based layer-by-layer hydrogel stacking. In fact, a skin-like structure was fabricated by laser-printing collagen-based NIH 3T3 fibroblast and human immortalized keratinocyte coatings that resulted in multilayered (with alternating cellular layers) structures [94]. The authors demonstrated the build-up of a basal lamina and the presence of cell-cell and cell-matrix junctions postprint as indications of the successful formation of skin-like tissue. Their multilayered approach utilized layer-by-layer stacking to mimic natural tissue with respect to cell density and distribution of different cell types and wherein the cells form native-like tissue.

4.5.3 *In situ* printing of bone substitute

Bone, like skin, is a relatively simple tissue. As such it is an enticing tissue to optimize 3D printing techniques. To that end, Keriquel et al. [95,96], have shown that laser-based bioprinting, due to its unprecedented cell-printing resolution and precision, is an attractive tool for the *in situ* printing of a bone substitute. Starting with a proof of concept and more recently expanding on their work, they have demonstrated the ability to utilize laser printing *in situ*. Specifically, the researchers deposited nano-hydroxyapatite (n-HA) and mesenchymal stromal cells into mouse calvaria defect of critical size, *in vivo*. Most important, direct laser irradiation on mouse duramater (using a similar exposure as for printing) showed that the 248 and 355 nm UV lasers used in the *in situ* experiments had no deleterious effects on the murine brain tissues studied. The results of this work demonstrated improved bone regeneration while not inducing a deleterious effect on the adjacent brain tissue.

Through the use of multiple ribbons with distinct cell types and/or bioinks, laser-based 3D printing offers a unique versatility. This bioprinting versatility,

demonstrated in bone, can be applied to other, more complex tissues, where additional components or cellular types may be organized in a specific 3D-arrangement that favors tissue regeneration. In addition, the noncontact nature of laser printing techniques makes it ideally suited for clinical application in the operating room. In the sterile environment of the operating room, automated printers could be directed by surgeons to achieve precise cellular deposition at the micron to millimeter scale.

4.5.4 *In vitro* research models

Biomimetic models are extremely valuable for the investigation of underlying cell and tissue mechanisms and the preclinical development of therapies. The precision of laser-based 3D-printing techniques allows for fabrication of many diverse *in vitro* models. Specifically, brain *in vitro* models are critically important for developing our understanding of basic nervous system cellular physiology, potential neurotoxic effects of chemicals, and specific cellular mechanisms of many disease states. In one case, Curley et al., utilized the technique to print primary mammalian neuronal cells, which is a sensitive and critical population. Although printing the cells led to a small drop in post-print viability compared to the breast cancer cell line, MDA-MB-231, the authors were still successful as a number (~85%) of rat embryonic dorsal root ganglion (DRG) cells survived, including neurons, and retained the ability to extend neuronal processes [97]. The ability to control numerous spatial aspects of the system, including print radius, print density, internodal distance, and geometric orientation of the DRG groups through laser-based printing demonstrates the flexibility of the modality and utilization of virtually any cell type. For example, Dauth et al. fabricated spheroids from groups of primary cells from separate brain regions and analyzed their interaction in a microfluidic chip fabricated by soft lithography. Therefore, a laser-based printing technique would allow for more precision in many aspects, including spheroid size and placement [98].

Just as in organ printing, a challenge in fabricating a truly biomimetic model for cancer or other disease (or healthy) process is replicating the vasculature. Phamduy et al. utilized a laser-based printing process to try and address this hurdle by printing on to live tissues (Figure 4.5) [82]. The authors took a rat mesentery model developed for studying angiogenesis [99] and printed cancer cells onto the living tissues to create an *ex vivo* experimental platform that enables time-lapse evaluation of cancer-cell dynamics during angiogenesis within a real microvascular network scenario. Of note, this research demonstrated the printing of cancer cells, with high precision, as single cells or as groups of cells, onto live rat connective tissues with intact microvascular networks. The work bridges the gap between expensive animal experiments and the misrepresentative or

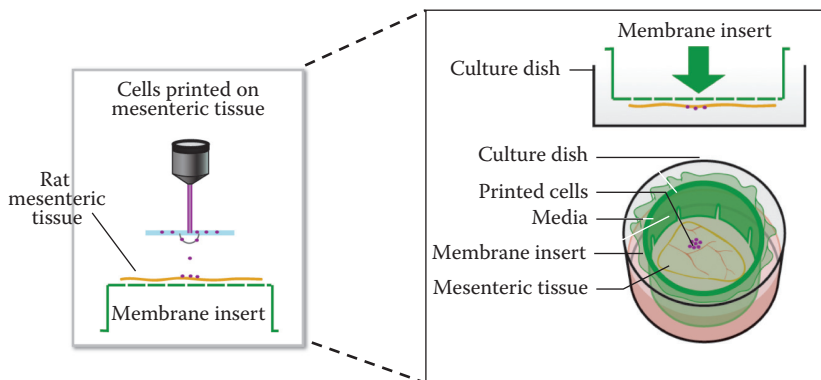


Figure 4.5 Laser-based cell printing on live tissue. Phamduy et al. utilized laser-based printing to deposit cancer cells onto rat mesenteric tissue to create a 3D-model for cancer-cell invasion. The tissue was harvested from adult rats and spread onto inverted commercially available transwell inserts. The authors demonstrated that bioprinted tissues can be cultured using normal culture conditions for at least up to 5 days. [82]

lacking *in vitro* models available for the study of complex, reciprocal cellular interactions between cancer cells, fibroblasts, blood vessels, and lymphatic vessels [82]. Although the authors utilized the platform for a specific purpose, their work demonstrates the ability of laser-based 3D-printing techniques to deposit onto live tissues as an avenue for creation of better research models.

4.6 Conclusion

In this chapter, we discussed laser-based 3D-printing techniques and their application to TE hurdles such as tissue perfusion. Several studies taken together demonstrate the capability of laser-based printing techniques to print a multitude of different biomaterials, including virtually all cell types. By utilizing the high resolution inherent in laser 3D printing, these biomaterials can be deposited in precise spatial arrangements that mimic natural tissue architecture.

Moving forward, TE and its associated disciplines will continue to stimulate the growth and development of laser-based 3D-bioprinting techniques. Future iterations of laser 3D printing must continue to adapt to the latest technology in automation, robotics, and biomaterials. Vascularized constructs created via techniques discussed herein are critical to the next stage of 3D-tissue fabrication, but future generations of laser 3D-bioprinting systems must be designed to address vascularization and beyond.

References

- Berthiaume F, Maguire TJ, Yarmush MI. Tissue engineering and regenerative medicine: History, progress, and challenges. *Ann. Rev. Chem. Biomol. Eng.* 2011, 2: 403–430.
- Atala A, Kasper FK, Mikos AG. Engineering complex tissues. *Sci. Transl. Med.* 2012, 4: 160rv12.
- Peng W, Unutmaz D, Ozbolat IT. Bioprinting towards physiologically relevant tissue models for pharmaceuticals. *Trends Biotechnol.* 2016, 34(9): 722–732.
- Badyalak SF, Nerem RM. Progress in tissue engineering and regenerative medicine. *Proc. Natl. Acad. Sci. USA* 2010, 107: 3285–3286.
- Radisic M, Malda J, Epping E, Geng W, Langer R, Vunjak-Novakovic G. Oxygen gradients correlate with cell density and cell viability in engineered cardiac tissue. *Biotechnol. Bioeng.* 2006, 93: 332–343.
- Okano T, Matsuda T. Muscular tissue engineering: Capillary-incorporated hybrid muscular tissues in vivo tissue culture. *Cell Transplant.* 1998, 7: 435–442.
- Sheridan MH, Shea LD, Peters MC, Mooney DJ. Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery. *J. Control. Release* 2000, 64: 91–102.
- Frerich B, Lindemann N, Kurtz-Hoffmann J, Oertel K. In vitro model of a vascular stroma for the engineering of vascularized tissues. *Int. J. Oral Maxillofac. Surg.* 2001, 30: 414–420.
- Bancroft GN, Sikavitsas VI, Mikos AG. Design of a flow perfusion bioreactor system for bone tissue-engineering applications. *Tissue Eng.* 2003, 9: 549–554.
- Harrison BS, Eberli D, Lee SJ, Atala A, Yoo JJ. Oxygen producing biomaterials for tissue regeneration. *Biomaterials* 2007, 28: 4628–4634.
- Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. *J. Bone Joint Surg. Am.* 2004, 86-A: 1541–1558.
- Malda JS, Klein TJ, Upton Z. The roles of hypoxia in the in vitro engineering of tissues. *Tissue Eng.* 2007, 13(9): 2153–2162.
- Marga F, Neagu A, Kosztin I, Forgacs G. Developmental biology and tissue engineering. *Birth Defects Res. C Embryo Today* 2007, 81:320–328.
- Mironov V, Kasyanov V, Nagy-Mehesz A, Moreno R, Hajdu Z, Trusk T, Ozolanta I et al. Tissue engineering of heart valve leaflet by self-assembly of tissue spheroids biofabricated from human fat tissue derived stem cells. In: Dössel O., Schlegel W.C. (Eds) *World Congress on Medical Physics and Biomedical Engineering*, September 7–12, 2009, Munich, Germany. IFMBE Proceedings, vol 25/10. Springer, Berlin, Germany.
- Khademhosseini A, Langer R, Borenstein J, Vacanti JP. Microscale technologies for tissue engineering and biology. *Proc. Natl. Acad. Sci. USA* 2006, 103: 2480–2487.
- Guillotin B, Guillemot F. Cell patterning technologies for organotypic tissue fabrication. *Trends Biotechnol.* 2011, 29: 183–190.
- Chrisey DB, Pique A, Fitz-Gerald J, Auyeung RCY, McGilla RA, Wua HD, Duignan M. New approach to laser direct writing active and passive mesoscopic circuit elements. *Appl. Surf. Sci.* 2000, 154: 593–600.
- Schiele NR, Corr DT, Huang Y, Raof NA, Xie Y, Chrisey DB. Laser-based direct-write techniques for cell printing. *Biofabrication* 2010, 2: 032001.
- Pique A, Kim H, Auyeung RCY, Beniam I, Breckenfeld E. Laser-induced forward transfer (LIFT) of congruent voxels. *Appl. Surf. Sci.* 2016, 374: 42–48.
- Hutmacher DW, Sittlinger M, Risbud, MV. Scaffold-based tissue engineering: Rationale for computer-aided design and solid free-form fabrication systems. *Trends Biotechnol.* 2004, 22: 354–362.

21. Mironov V, Forgacs G. Bioprinting living structures. *J. Mater. Chem.* 2007, 17: 2054–2060.
22. Ozbolat IT, Hospodiuk M. Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials* 2016, 76: 321–343.
23. Gruene M, Phlaum M, Hess C, Diamantouros S, Schlie S, Deiwick A, Koch L et al. Laser printing of three-dimensional multicellular arrays for studies of cell-cell and cell-environment interactions. *Tissue Eng. Part C* 2011, 17: 973–982.
24. Lin Y, Huang Y, Chrisey DB. Droplet formation in matrix-assisted pulsed-laser evaporation direct writing of glycerol-water solution. *J. Appl. Phys.* 2009, 105 (9): 093111.
25. Nishimura N, Schaffer CB, Friedman B, Lyden PD, Kleinfeld D. Penetrating arterioles are a bottleneck in the perfusion of neocortex. *Proc. Natl. Acad. Sci. USA* 2007, 104: 365–370.
26. Schiele NR, Chrisey DB, Corr DT. Gelatin-based laser direct-write technique for the precise spatial patterning of cells. *Tissue Eng. C* 2011, 17: 289–298.
27. Phamduy TB, Raof NA, Schiele NR, Yan Z, Corr DT, Huang Y, Xie Y, Chrisey DB. Laser direct-write of single microbeads into spatially-ordered patterns. *Biofabrication* 2012, 4: 025006.
28. Fogarassy E, Fuchs C, Kerherve F, Hauchecorne G, Perriere J. Laser-induced forward transfer—a new approach for the deposition of high-Tc superconducting thin-films. *J. Mater. Res.* 1989, 4: 1082–1086.
29. Piqué A, Chrisey DB, Auyeung RCY, Fitz-Gerald J, Wu HD, McGill RA, Lakeou S, Wu PK, Nguyen V, Duignan M. A novel laser transfer process for direct writing of electronic and sensor materials. *Appl. Phys. A: Mater. Sci. Process* 1999, 69(7): S279–S284.
30. Fitz-Gerald JM, Chrisey DB, Pique A, Auyeung RCY, Mohdi R, Young HD, Wu HD, Lakeou S, Chung R. Matrix assisted pulsed laser evaporation direct write (MAPLE DW): A new method to rapidly prototype active and passive electronic circuit elements. *Mat. Res. Soc. Proc.* 2000, 625: 99–110.
31. Chrisey DB. Materials processing: The power of direct writing. *Science* 2000, 289 (5481): 879–881.
32. Patz TM, Doraiswamy A, Narayan RJ, He W, Zhong Y, Bellamkonda R, Modi R, Chrisey DB. Three-dimensional direct writing of B35 neuronal cells. *J. Biomed. Mater. Res. B* 2006, 78: 124–130.
33. Fernandez-Pradas JM, Colina M, Serra P, Dominguez J, Morenza JL. Laser-induced forward transfer of biomolecules. *Thin Solid Films* 2004, 453–454: 27–30.
34. Duocastella M, Colina M, Fernandez-Pradas JM, Serra P, Morenza JL. Study of the laser-induced forward transfer of liquids for laser bioprinting. *Appl. Surf. Sci.* 2007, 253: 7855–7859.
35. Dinca V, Farsari M, Kafetzopoulos D, Popescu A, Dinescu M, Fotakis C. Patterning parameters for biomolecules microarrays constructed with nanosecond and femtosecond UV lasers. *Thin Solid Films* 2008, 516: 6504–6511.
36. Koch L, Kuhn S, Sorg H, Gruene M, Schlie S, Gaebel R, Polchow B et al. Laser printing of skin cells and human stem cells. *Tissue Eng. C Methods* 2010, 16(5): 847–854.
37. Ovsianikov A, Gruene M, Pflaum M, Koch L, Maiorana F, Wilhelm M, Haverich A, Chichkov B. Laser printing of cells into 3D scaffolds. *Biofabrication* 2010, 2: 014104.
38. Arnold CB, Serra P, Pique A. Laser direct-write techniques for printing of complex materials. *MRS Bull.* 2007, 32: 23–31.
39. Hopp B, Smausz T, Antal Z, Kresz N, Bor Z, Chrisey D. Absorbing film assisted laser induced forward transfer of fungi (*Trichoderma conidia*). *J. Appl. Phys.* 2004, 96: 3478–3481.

40. Hopp B, Smausz T, Kresz N, Barna N, Bor Z, Kolozsvári L, Chrisey DB, Szabó A, Nógrádi A. Survival and proliferative ability of various living cell types after laser-induced forward transfer. *Tissue Eng.* 2005, 11: 1817–1823.
41. Smausz T, Hopp B, Kecskemeti G, Bor Z. Study on metal microparticle content of the material transferred with absorbing film assisted laser induced forward transfer when using silver absorbing layer. *Appl. Surf. Sci.* 2006, 252: 4738–4742.
42. Barron JA, Wu P, Ladouceur HD, Ringeisen BR. Biological laser printing: A novel technique for creating heterogeneous 3-dimensional cell patterns. *Biomed. Microdevices* 2004, 6: 139–147.
43. Barron JA, Krizman DB, Ringeisen BR. Laser printing of single cells: Statistical analysis, cell viability, and stress. *Ann. Biomed. Eng.* 2005, 33: 121–130.
44. Chen CY, Barron JA, Ringeisen BR. Cell patterning without chemical surface modification: Cell-cell interactions between printed bovine aortic endothelial cells (BAEC) on a homogeneous cell-adherent hydrogel. *Appl. Surf. Sci.* 2006, 252: 8641–8645.
45. Othon CM, Wu XJ, Anders JJ, Ringeisen BR. Single-cell printing to form three-dimensional lines of olfactory ensheathing cells. *Biomed. Mater.* 2008, 3: 034101.
46. Barron JA, Ringeisen BR, Kim HS, Spargo BJ, Chrisey DB. Application of laser printing to mammalian cells. *Thin Solid Films* 2004, 453–454: 383–387.
47. Chrisey DB, Pique A, McGill RA, Horwitz JS, Bubbs DM, Wu PK, Ringeisen BR. Laser deposition of polymer and biomaterial films. *Chem. Rev.* 2003, 103: 553–576.
48. Ringeisen BR, Kim H, Barron JA, Krizman DB, Chrisey DB, Jackman S, Auyeung RY, Spargo BJ. Laser printing of pluripotent embryonal carcinoma cells. *Tissue Eng.* 2004, 10: 483–491.
49. Doraiswamy A, Narayana RJ, Lippert T, Urech L, Wokaun A, Nagelc M, Hopp B, Dinescue M, Modi R, Auyeung RCY, Chrisey DB. Excimer laser forward transfer of mammalian cells using a novel triazene absorbing layer. *Appl. Surf. Sci.* 2006, 252: 4743–4747.
50. Pirlo RK, Wu P, Liu J, Ringeisen B. PLGA/hydrogel biopapers as a stackable substrate for printing HUVEC networks via BioLp™. *Biotechnol. Bioeng.* 2012, 109: 262–273.
51. Riggs BC, Dias AD, Schiele NR, Cristescu R, Huang Y, Corr DT, Chrisey DB. Matrix-assisted pulsed laser methods for biofabrication. *MRS Bull.* 2011, 36: 1043–1050.
52. Wu PK and Ringeisen BR. Development of human umbilical vein endothelial cell (huvec) and human umbilical vein smooth muscle cell (huvsmc) branch/stem structures on hydrogel layers via biological laser printing (biolp). *Biofabrication* 2010, 2: 014111.
53. Gaebel R, Ma N, Liu J, Guan J, Koch L, Klopsch C, Gruene M, Toelk A, Wang W, Mark P, Wang F, Chichkov B, Li W, Steinhoff G. Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials* 2011, 32: 9218–9230.
54. Pirlo RK, Wu PK, Ringeisen BR. Computer aided design and manufacturing of soft, three-dimensional, multilayer, biological constructs via laser printing onto laser machined composite biopapers. *J. Imaging Sci. Technol.* 2014, 58(4): 040401.
55. Dias AD, Kingsley DM, Corr DT. 2015. Engineering 2D and 3D cellular microenvironments using laser direct-write. In: Grace L, Fisher J, Leong K, (Eds.) *3D Bioprinting Nanotechnology in Tissue Engineering and Regenerative Medicine*. London, UK: Elsevier. pp. 105–127.
56. Vinson BT, Phamduy TB, Shipman J, Riggs B, Strong AL, Sklare SC, Murfee WL et al. Laser direct-write based fabrication of a spatially-defined, biomimetic construct as a potential model for breast cancer cell invasion into adipose tissue. *Biofabrication* 2017, 9: 025013.

57. Griffith CK, Miller C, Sainson RCA, Calvert JW, Jeon NL, Hughes CC, George SC. Diffusion limits of an in vitro thick prevascularized tissue. *Tissue Eng.* 2005, 11: 257–266.
58. Freed LE, Marquis JC, Langer R, Vunjak-Novakovic G. Kinetics of chondrocyte growth in cell-polymer implants. *Biotechnol. Bioeng.* 1994, 43: 597–604.
59. Du Y, Lo E, Ali S, Khademhosseini A. Directed assembly of cell-laden microgels for fabrication of 3D tissue constructs. *Proc. Natl. Acad. Sci. USA* 2008, 105(28): 9522–9527.
60. King KR, Wang CCJ, Kaazempur-Mofrad MR, Vacanti JP, Borenstein JT. Biodegradable microfluidics. *Adv. Mater.* 2004, 16: 2007–2012.
61. Kachouie NN, Du Y, Bae H, Khabiry M, Ahari AF, Zamanian B, Fukuda J, Khademhosseini A. Directed assembly of cell-laden hydrogels for engineering functional tissues. *Organogenesis* 2010, 6: 234–244.
62. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. *Science* 1980, 210: 908–910.
63. Kingsley DM, Dias AD, Corr DT. Microcapsules and 3D customizable shelled micro-environments from laser direct-written microbeads. *Biotechnol. Bioeng.* 2016, 113(22): 2264–2274.
64. Wang W, Liu X, Xie Y, Zhang H, Yu W, Xiong Y, Xie W, Ma X. Microencapsulation using natural polysaccharides for drug delivery and cell implantation. *J. Mater. Chem.* 2006, 16: 3252.
65. Zhang X, Wang W, Yu W, Xie Y, Zhang X, Zhang Y, Ma X. Development of an in vitro multicellular tumor spheroid model using microencapsulation and its application in anti-cancer drug screening and testing. *Biotechnol. Prog.* 2005, 21: 1289–1296.
66. Hernandez RMA, Orive G, Murua A, Pedraz JL. Microcapsules and microcarriers for in situ cell delivery. *Adv. Drug Deliv. Rev.* 2010, 62:711–730.
67. Thu B, Bruheim P, Espevik T, Smidsrød O, Soon-Shiong P, Skjåk-Braek G. Alginate polycation microcapsules. II. Some functional properties. *Biomaterials* 1996, 17: 1069–1079.
68. Gåserød O, Smidsrød O, Skjåk-Braek G. Microcapsules of alginate-chitosan-I: A quantitative study of the interaction between alginate and chitosan. *Biomaterials* 1998, 19:1815–1825.
69. Lin RZ, Chu WC, Chiang CC, Lai CH, Chang HY. Magnetic reconstruction of three-dimensional tissues from Multicellular Spheroids. *Tissue Eng. Part C Methods* 2008, 14(3): 197–205.
70. Kelm JM, Djonov V, Ittner LM, Fluri D, Born W, Hoerstrup SP, Fussenegger M. Design of custom-shaped vascularized tissues using microtissue spheroids as minimal building units. *Tissue Eng.* 2006, 12(8): 2151–2160.
71. Jakob K, Neagu A, Mironov V, Markwald RR, Forgacs G. Engineering biological structures of prescribed shape using self-assembling multicellular systems. *Proc. Natl. Acad. Sci. USA* 2004, 101(9): 2864–2869.
72. Vrij E, Rouwkema J, LaPointe V, van Blitterswijk C, Truckenmuller R, Rivron N. Directed assembly and development of material-free tissues with complex architectures. *Adv. Mater.* 2016, 28: 4032–4039.
73. Guven S, Chen P, Inci F, Tasoglu S, Erkmen B, Demirci U. Multiscale assembly for tissue engineering and regenerative medicine. *Trends Biotechnol.* 2015, 33: 269–279.
74. Susienka MJ, Wilks BT, Morgan JR. Quantifying the kinetics and morphological changes of the fusion of spheroid building blocks. *Biofabrication* 2016, 8: 045003.
75. Schiele NR, Koppes RA, Corr DT, Ellison KS, Thompson DM, Ligon LA, Lippert TKM, Chrisey DB. Laser direct writing of combinatorial libraries of idealized cellular constructs: Biomedical applications. *Appl. Surf. Sci.* 2009, 255: 5444–5447.

76. Dias AD, Unser AM, Xie Y, Chrisey DB, Corr DT. Generating size-controlled embryoid bodies using laser direct-write. *Biofabrication* 2014, 6: 025007.
77. Ringeisen BR, Pirlo RK, Wu PK, Boland T, Huang Y, Sun W, Hamid Q, Chrisey DB. Cell and organ printing turns 15: Diverse research to commercial transitions. *MRS Bull.* 2013, 38: 834–843.
78. Yan J, Huang Y, Chrisey DB. Laser-assisted printing of alginate long tubes and annular constructs. *Biofabrication* 2013, 5: 015002.
79. Xiong R, Zhang Z, Chai W, Huang Y, Chrisey DB. Freeform drop-on-demand laser printing of 3D alginate and cellular constructs. *Biofabrication* 2015, 7: 045011.
80. Xiong R, Zhang Z, Huang Y. Identification of optimal printing conditions for laser printing of alginate tubular constructs. *J. Manuf. Process.* 2015, 20: 450–455.
81. Christensen K, Xu C, Chai W, Zhang Z, Fu J, Huang Y. Freeform inkjet printing of cellular structures with bifurcations. *Biotechnol. Bioeng.* 2015, 112: 1047–1055.
82. Phamduy TB, Sweat RS, Azimi MS, Burow ME, Murfee WL, Chrisey DB. Printing cancer cells into intact microvascular networks: A model for investigating cancer cell dynamics during angiogenesis. *Integrative Biol.* 2015, 7: 1068–1078.
83. Boland T, Xu T, Damon B, Cui X. Application of inkjet printing to tissue engineering. *Biotechnol. J.* 2006, 1: 910–917.
84. Nishiyama Y, Nakamura M, Henmi C, Yamaguchi K, Mochizuki S, Nakagawa H, Takiura K. Development of a three-dimensional bioprinter: Construction of cell supporting structures using hydrogel and state-of-the-art inkjet technology. *J. Biomech. Eng.* 2009, 131: 035001.
85. Norotte C, Marga FS, Niklason LE, Forgacs G. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials* 2009, 30: 5910–5917.
86. Skardal A, Zhang J, Prestwich GD. Bioprinting vessel-like constructs using hyaluronan hydrogels crosslinked with tetrahedral polyethylene glycol tetracrylates. *Biomaterials* 2012, 31(24): 6173–6181.
87. Miller JS, Stevens KR, Yang MT, Baker BM, Nguyen DH, Cohen DM, Toro E et al. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nat. Mater.* 2012, 11: 768–774.
88. Xu C, Chai W, Huang Y, Markwald RR. Scaffold-free inkjet printing of three-dimensional zigzag cellular tubes. *Biotechnol. Bioeng.* 2012, 109: 3152–3160.
89. Kolesky DB, Truby RL, Gladman AS, Busbee TA, Homan KA, Lewis JA. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv. Mater.* 2014, 26: 3124–3130.
90. Laschke MW, Menger MD. Prevascularization in tissue engineering: Current concepts and future directions. *Biotechnol. Adv.* 2016, 34(2): 112–121.
91. Utzinger U, Baggett B, Weiss JA, Hoying JB, Edgar LT. Large-scale time series microscopy of neovessel growth during angiogenesis. *Angiogenesis* 2015, 18: 219–232.
92. Laschke MW, Menger MD. Vascularization in tissue engineering: Angiogenesis versus inosculation. *Eur. Surg. Res.* 2012, 48: 85–92.
93. Eweida AM, Nabawi AS, Marei MK, Khalil MR, Elhammady HA. Mandibular reconstruction using an axially vascularized tissue-engineered construct. *Ann. Surg. Innov. Res.* 2011, 5: 2.
94. Koch L, Deiwick A, Schlie S, Michael S, Gruene M, Coger V, Zychlinski D et al. Skin tissue generation by laser cell printing. *Biotechnol. Bioeng.* 2012, 109: 1855–1863.
95. Keriquel V, Guillemot F, Arnault I, Guillotin B, Miraux S, Amédée J, Fricain JC, Catros S. In vivo bioprinting for computer- and robotic-assisted medical intervention: Preliminary study in mice. *Biofabrication* 2010, 2: 014101.

96. Keriquel V, Oliviera H, Remy M, Ziane S, Delmond S, Rousseau B, Rey S et al. In situ printing of mesenchymal stromal cells, by laser-assisted bioprinting, for in vivo bone regeneration applications. *Sci. Rep.* 2017, 7: 1778.
97. Curley JL, Sklare SC, Bowser DA, Saksena J, Moore MJ, Chrisey DB. Isolated node engineering of neuronal systems using laser direct write. *Biofabrication* 2016, 8: 015013.
98. Dauth S, Maoz BM, Sheehy SP, Hemphill MA, Murty T, Macedonia MK, Greer AM, Budnik B, Parker KK. Neurons derived from different brain regions are inherently different in vitro: A novel multiregional brain-on-a-chip. *J. Neurophysiol.* 2017, 117: 1320–1341.
99. Stapor PC, Azimi MS, Ahsan T, Murfee WL. An angiogenesis model for investigating multicellular interactions across intact microvascular networks. *Am. J. Physiol.: Heart Circ. Physiol.* 2013, 304: H235–H245.

Chapter 5 Inkjet-based 3D bioprinting

Shibu Chameettachal
and Falguni Pati

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5.1 Introduction

Three-dimensional (3D) bioprinting techniques, developed over the past two decades, have the potential to fabricate living, patient-specific tissues and organs for use in regenerative medicine (Kesti et al. 2016). Inkjet-based bioprinting is one of the oldest yet promising technologies, which combines the solid free-form

fabrication process and precise cell placement in 2D and 3D. Typically, inkjet printing converts digital data of an image or character from a computer and reproduces it on to a material or substrate using ink drops in a noncontact mode (Mohebi and Evans 2002). In addition to its wide application in automation office tools, it has also been widely used in microengineering for printing electronic materials (Sirringhaus et al. 2000). Inkjet printer deposits controlled volume of ink in a layer-by-layer fashion to predefined locations on successive layers to form a 3D construct (Xu et al. 2008). Drop-on-demand (DOD) printers, a commonly used inkjet printer, are used for biological and nonbiological applications. The idea of printing of biological substances was introduced by Klebe (1988) after using HP thermal DOD inkjet printer to deposit collagen and fibronectin in 1988. Later on, Thomas Boland customized the thermal inkjet printer (TIJ) for printing living cells in viable state (Wilson and Boland 2003). After this, scientists have made great progress in patterning molecules such as DNA, depositing proteins (Miller et al. 2006, Ilkhanizadeh et al. 2007, Miller et al. 2009), and printing tissues and organs by inkjet printing (Okamoto et al. 2000). The current focus of inkjet bioprinting is fabrication of 3D structures with clinically significant heights to extend their effectiveness in the direction of clinical applications. In this chapter, we discuss about the principle of inkjet bioprinting technology, types of inkjet printers in use, materials used for printing, and the critical parameters involved in this technique. Furthermore, challenges associated with techniques and the future perspectives are discussed.

5.2 Working principle

The basic mechanism of an inkjet printer is that the bioink is ejected out as droplets from the print head on a receiving substrate as a result of gravity, pressure, and fluid mechanics (Saunders and Derby 2014). The first attempt of inkjet bioprinting was done with a modified HP printer, where the paper was replaced with electronically controlled elevator stage that can control the *z-axis*, and the cartridge is replaced with a biological material (Arrabito et al. 2009, Cui et al. 2013). Inkjet bioprinters are customized to print cells or biological materials at high resolution, precision and speed. Additionally, they are also equipped with movements in “*x*”, “*y*” directions and a third dimension “*z*”, that is for height. (Figure 5.1). Based on the different modalities employed, inkjet printing or droplet-based bioprinting (DBB) is classified into three types, and they are (1) continuous inkjet (CIJ) printing, where a stream of droplets is generated under air pressure, (2) DOD, the widely used printer where the printer generates drops only when it is required, and (3) electrohydrodynamic jet (EHD) bioprinting, where droplets are ejected with the help of high-electric voltage (Gudapati et al. 2016).

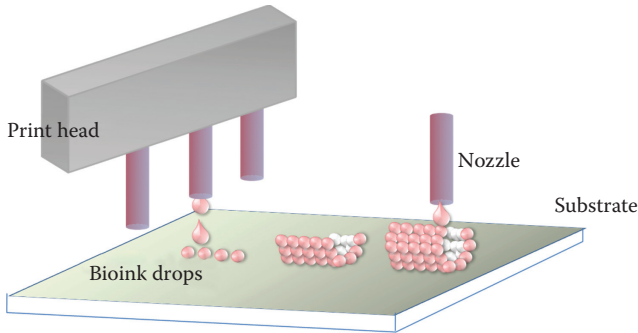


Figure 5.1 Inkjet bioprinting system in which a stream of liquid transforms into a trail of discrete drops to fabricate the tissue by placing the drops layer-by-layer.

5.2.1 Continuous inkjet printing

CIJ exploits the natural propensity of a stream of liquid to transform into a trail of discrete drops (the Rayleigh–Plateau instability) through morphological transformation and is generally twice the size of the orifices. Rayleigh–Plateau instability has been described (Rayleigh 1878) as a cylindrical volume of liquid jet that gets agitated by several factors including but not limited to the kinetic energy motion of the jet and the potential energy due to surface energy of the jet (Figure 5.2). When the wavelength of the perturbed jet exceeds its initial radius by a certain limit as a function of number of waves per unit-length, wave number (k) and the initial jet radius (R_0) are less than 1 ($kR_0 < 1$), the perturbation grows exponentially and the jet gets distorted to a stream of droplets

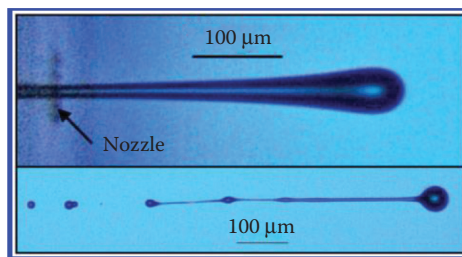


Figure 5.2 High-speed photograph of a drop following detachment from the nozzle. Here, the ligament is breaking up to form satellite drops. (Reproduced from Saunders, R.E. and Derby, B., *Int. Mat. Rev.*, 59, 430–448, 2014. With permission.)

by minimizing its potential energy. As the ink is conductive to electricity, the drops get charged immediately after they form and are guided by electric or magnetic fields to their desired locations (Okamoto et al. 2000).

5.2.2 Drop-on-demand inkjet printer

In a DOD printer, the placement of a drop is accomplished by propelling bioink to a desired location through the nozzle that was previously positioned and is followed by ejection through the nozzle. DOD inkjet printer can achieve smaller drop volumes greater image resolution even at lower frequencies and printed drops are comparable to the orifice diameter (Technote 1999, Saunders and Derby 2014). There are mainly three types of inkjet printing and they are explained as follows.

5.2.2.1 Thermal inkjet printers The TIJ are equipped with electrically heated print heads that can achieve temperatures ranging from 200°C to 300°C and typically operate for $\sim 2 \mu\text{s}$, producing produces a vapor bubble (Figure 5.3). Subsequently, this bubble starts to expand as a function of heat, explodes which generates pulses of pressure, this helps in overcoming the surface tension force at the tip of the nozzle, and ejects droplets (Figure 5.4). It is noteworthy that it happens without having any detrimental effect on either the stability of biological molecules, such as DNA (Goldmann and Gonzalez 2000, Okamoto et al. 2000), the viability of the cells, and/or the functionality of the printed mammalian cells (Xu et al. 2005, 2006). This short period of heating results in an increase of only 4°C–10°C in temperature in the printer head (Cui et al. 2010). TIJs have some advantages such as wide availability, high print speed, and low cost. However, the risk of exposing materials and cells to mechanical and thermal stress is making it a risky process. Apart from that, unsteady or varying droplet sizes, reduced droplet directionality, frequent clogging of the nozzles, and unreliable

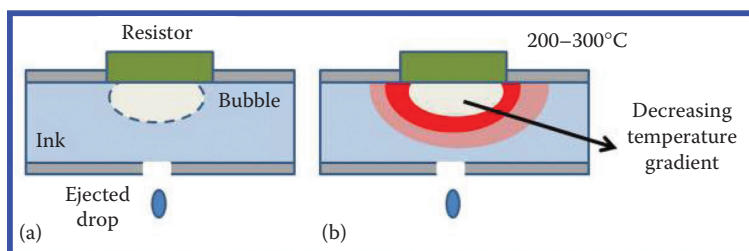


Figure 5.3 (a) The resistor superheats the fluid and creates a bubble, which expands and expels the drop. (b) Schematic of the temperature gradient in a thermal inkjet bulk fluid experiences lower temperatures than the fluid localized at the resistor. (Reproduced from Saunders, R.E. and Derby, B., *Int. Mat. Rev.*, 59, 430–448, 2014. With permission.)

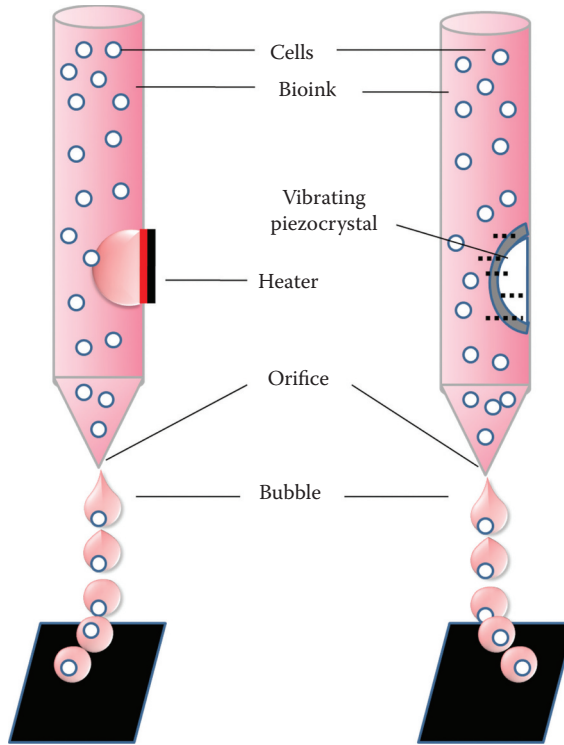


Figure 5.4 Nozzle assembly containing bioink of thermal and piezoelectric bioprinting systems, showing inserted heater and piezocrystal, respectively.

cell encapsulation are some of the significant disadvantages for the use of TIJ printers in 3D bioprinting (Murphy and Atala 2014).

5.2.2.2 Piezoelectric inkjet printing A variant of inkjet printer uses piezoelectric crystals inside the print head to create an acoustic wave to break the liquid bioink into droplets. Applied pulses of voltage to a piezoelectric material induce a rapid change in shape of the actuators with a sudden change in the volume of the ink chamber and generate the pressure waves needed to eject droplets from the nozzle at regular intervals (Figure 5.4) (Tekin et al. 2008). Few groups have optimized the process parameters such as bioink compositions (Pataky et al. 2012); mode of actuation of piezoelectric element (Yamaguchi et al. 2012); and also the echo time, amplitude, rise and fall time, and frequency and dwell time (Xu et al. 2012). Other inkjet printers associated with an ultrasound field use an acoustic radiation force to eject liquid droplets from the orifice (Demirci and Montesano 2007, Fang et al. 2012). Acoustic inkjet printers can generate controlled droplet size, ejection directionality, ability to generate uniform size of droplets, and

advantage of devoid of pressure stressors and exposure of cells to heat (Nakamura et al. 2006, Saunders et al. 2008, Tibbitt and Anseth 2009). In addition, by usage of an open-pool nozzle-less ejection system (Mehta et al. 2011), it reduces nozzle clogging, loss of viability, and cellular function as there is no shear stress applied on cells at the nozzle tip wall. In acoustic ejectors, multiple cell types and materials can be co-printed simultaneously as it is equipped with multiple ejectors (Tasoglu and Demirci 2013). However, the high frequencies (15–25 kHz) are a major concern as they can cause damage to the cells by disrupting the cell membrane and can induce cell lysis (Cui et al. 2012a).

5.2.2.3 Electrostatic inkjet bioprinters Electrostatic bioprinters are another type of inkjet printers, where they generate droplets by a minor enlargement in the volume of the ink chamber or ink solution by means of a pressure plate. During application of a pulse of voltage between the plates, it deflects and regains the shape in the absence of applied voltage. Due to this, droplets are formed and ejected out from the nozzle. This kind of inkjet printer was successfully used by Nishiyama et al. (2008) for printing cellular and acellular materials.

5.2.3 Electrohydrodynamic jet bioprinting

In the EHD bioprinting technique, the electric field is the pulling force, which obviates the substantial high pressure to push the bioink through the orifice (Onses et al. 2015). The EHD bioprinters' nozzles have very small orifice diameter ($\leq 100 \mu\text{m}$) and highly concentrated bioinks in which up to 20% W/V can be printed (Jayasinghe et al. 2006). In EHD, the fed bioink through a nozzle takes a shape of sphere-shaped meniscus at the nozzle tip owing to the surface tension (Poellmann et al. 2011). An applied high voltage (0.5–20 kV) between substrate and nozzle generates an electric field between the nozzle and the substrate (Hayati et al. 1986, Gasperini et al. 2014). Thus, developed electric field builds up mobile ions in the bioink near the surface of the bioink meniscus suspended at the tip. Subsequently, the meniscus is deformed to a conical shape called a "Taylor cone" due to the electrostatic repulsions between the ions in the bioink. Consequently, the built-up electrostatic forces at the orifice overcome the surface tension of the bioink droplet and the droplets are ejected from the nozzle. This phenomenon is dependent on the applied voltage and on different process parameters. Jetting mode (Kim et al. 2007, Onses et al. 2015) and cell viability (Workman et al. 2014) are determined by the parameters such as the applied voltage (strength of the electric field), properties of the bioink including concentration, cell types, and the flow rate of bioink (Jayasinghe and Townsend-Nicholson 2006). A continuous uninterrupted stream of the bioink creates a continuous Taylor cone and is known as cone-jet-mode, which is observed only at high voltages, whereas low voltages and flow rates result in dripping mode at the same time (Gudapati et al. 2016). Voltages and/or flow rates at an intermediate level showed distinct droplets (Gudapati et al. 2016).

5.3 Materials in use and biofunctionality

For bioprinting applications in general, materials must retain a considerable amount of water (e.g., hydrogels), in which case the most important deposition sequence will be wet-on-wet drop interaction. The surface tension force exerted on the sessile liquid drops regulates this wet-on-wet drop adhesion. Characteristics such as biocompatibility, low viscosity, increased cell adhesive properties, bioprintability, and suitable biodegradability along with high mechanical strength limit the choice of suitable biomaterials for DBB. Hence, only a limited range of hydrogels including fibrin, alginate, collagen, polyethylene glycol (PEG), and methacrylated gelatin (GelMA) have been used (Gudapati et al. 2016). In general, during printing the hydrogels should be in liquid form to facilitate the flow and after printing, precursor hydrogel polymers should be solidified by gelation or cross-linking process such as temperature-sensitive phase transition, enzymatic cross-linking, and photocross-linking or by altering pH. Hydrogels of natural origin superiorly support biological functions, whereas synthetic polymers are beneficial in terms of material functionalization or manipulation, cost efficiency, and reproducibility (Lee et al. 2015). Some of the successful examples of inkjet printing by using a variety of materials are discussed in the following.

5.3.1 Alginate

Ashley et al. adopted a two-step gelation process for the printing of silk fibroin, which utilizes alginate as a sacrificial hydrogel during an inkjet-based printing process. A blend of alginate and silk fibroin along with NIH 3T3 fibroblasts is utilized to achieve rapid gelation by calcium alginate formation during printing, followed by horseradish peroxidase (HRP)-catalyzed covalent cross-linking of the fibroin protein. The constructs were intact after the calcium chelation to liquefy the alginate shows the formation of silk fibroin hydrogel. Cell proliferation and spreading in the hydrogel were also noticed (Compaan et al. 2016).

Shu's group used sodium alginate with hepatocytes derived from human-induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) as bioink to print with a microvalve bioprinter, a class of DBB to fabricate 3D liver tissue models (Faulkner-Jones et al. 2015). In this study, alternate layers of the bioink and calcium chloride cross-linker were co-printed to facilitate the alginate cross-linking. After 17 days of post-bioprinting, cells were differentiated and hepatic markers such as hepatocyte nuclear factor 4 alpha (HNF4a), a transcription factor that regulates the expression of hepatic genes, were demonstrated along with albumin.

Atala's group used TIJ bioprinting technique to fabricate 3D heterogeneous tissue constructs with multiple cell types viz human amniotic fluid-derived stem cells (hAFSCs), canine smooth muscle cells (dSMCs), and bovine aortic endothelial cells (bECs). dSMCs mixed with CaCl_2 cross-linker were bioprinted into

a sodium alginate collagen solution. Printing successive layers of cell-laden solution achieved the targeted final pie shape with three different cell types. They also demonstrated that bioprinted cells could differentiate and maintain their functionality both *in vitro* and *in vivo* (Xu et al. 2013b).

5.3.2 Collagen type I, fibrin and thrombin

In another study by Atala's group, microvalve bioprinting and electrospinning were employed to engineer hybrid cartilage tissue. A bioink solution of collagen and fibrinogen with chondrocytes was electrospun into layers of poly- ϵ -caprolactone (PCL) fibers that were printed previously. In addition, thrombin was bioprinted in alternating layer as cross-linker. The characterization studies revealed that cartilage constructs maintained their biological functions both *in vitro* and *in vivo*. In comparison with the cartilage constructs without PCL fibers, constructs with incorporated electrospun PCL fibers showed enhanced biological and mechanical characteristics (Xu et al. 2013a). As an alternative to bioprinting followed by implantation, *in situ* bioprinting was introduced where cells, extracellular matrix, or other engineered proteins were bioprinted directly into lesion sites (Cui et al. 2012b). In a study, cells with biological materials were bioprinted over surgical skin wounds on a nude mouse of size 2.0×2.0 cm using a microvalve bioprinter. Three layers of solution and two layers with 5×10^6 AFSCs or SMCs in collagen-fibrinogen solution and thrombin cross-linker were alternatively (2 and 3 layers, respectively) bioprinted over each wound and the results demonstrated that AFSCs were comparable to mesenchymal stem cells (MSCs) in skin regeneration and *in situ* bioprinting of cells accelerated the process of wound healing (Skardal et al. 2012).

5.3.3 Methacrylated gelatin

With a modified TIJ, Kim et al. printed polyethylene glycol dimethacrylate (PEGDMA) and human articular chondrocytes into a cartilage defect area within an osteochondral plug in layer-by-layer fashion. A representative cartilage defect with 4 mm diameter and 2 mm thickness was fabricated with anatomic cartilage zonal structures by Cui et al using modified HP Deskjet 500 printer. They printed the structure layer-by-layer with $0.23 \mu\text{l}$ polyethylene glycol dimethacrylate (PEGDMA) bioink containing 1140 human chondrocytes (5×10^6 cells/ml) in each layer. The volume of bioink to prepare each zone was about 18 ml. Each layer was photopolymerized by using UV and the structure showed viability of $89.2\% \pm 3.6\%$ (Cui et al. 2012).

5.3.4 Polyethylene glycol

Gao et al. (2015) demonstrated simplified steps of conjugation of peptide, fabrication of scaffold, precise cell delivery by coprinting with in-house developed acrylated peptides and acrylated polyethylene glycol (PEG) hydrogel with

instantaneous photopolymerization in an inkjet printer. Bone marrow-derived human hMSCs were printed precisely with minimal simultaneous UV exposure and this showed $87.9\% \pm 5.3\%$ cell viability. This peptide-conjugated PEG scaffold demonstrated enhanced bone and cartilage differentiation and biocompatibility. In addition, the construct was mechanically superior without letting go of the water-like property for continuous inkjet bioprinting. The reported 500 kPa compressive modulus of the printed PEG hydrogel was 100 times higher than the reported compressive modulus of natural hydrogels (Spalazzi et al. 2008, Khanarian et al. 2011).

Though many hydrogel polymers can be dispensed through inkjet-based printing systems, certain materials are not suitable for these systems due to the limitation of printing mechanism. Vibrations, bubbles, pneumatic pressure less than 10 psi, and mild ejecting mechanisms, which minimize cell death, alteration in the cellular phenotype, or loss of functionality after printing are considered as major advantages in viable cell printing. On the other hand, printer head clogging and irregular printing patterns due to highly viscous materials and usage of increased cell density suspensions are the limitations. In general, microvalve-based bioprinters have advantages in handling viscous materials over piezoelectric or thermal bioprinters as the additional pressure applied on the loaded materials can be tuned to obtain proper ejecting forces (Lee et al. 2015).

5.4 Critical process parameters

To generate a consistent patterning method and functionality, it is necessary to understand the printing dynamics. This can be influenced by many factors and each factor is dependent on each other or influenced by other parameters. It includes resolution, orifice size, matrix composition, and viscosity. These factors will be decisive in sedimentation, droplet size, cell aggregation, cell patterning from precision cell placement to the printing of a defined area, cellular viability, and the overall functionality of the construct.

5.4.1 Resolution

In inkjet-based printers, patterns are made in drop-by-drop and a series of droplets are closely deposited and they merge to form line or surface patterns (Lee et al. 2015). Resolution down to a nanometer may be difficult for a bioprinter to accomplish, but high resolution is an advantage for biofabrication of 3D-complex structures. The ultimate resolution of an inkjet bioprinter is mainly dependent on the print head used for that particular application. Specifically, the minimum droplet size that the printer can generate depends on the type of print head (Lee et al. 2015). The final resolution of the bioprinted construct is the resolution after the

droplet spreads on the surface and this is different from the print head-dependent resolution. The actual resolution of inkjet-based printer tends to be lower than the minimum droplet size because the contour of printed pattern often becomes enlarged by merging of closely printed droplets. For example, pressure-driven microvalves deliver approximately 500 cells per droplet and spread over 1 mm on the bioprinting surface. Pressure-driven microvalve systems in general has lower spatial resolution but exhibits superior throughput than piezoelectric or thermal print heads and are the effortless type of print head, which can be incorporated into a custom device (Binder et al. 2011).

5.4.2 Orifice size

Typically, the orifice size that is smaller than the cell size is unsuitable for cell bioprinting with a high resolution. Precision at the single-cell level is dependent on the average drop volume, which in turn is determined by the orifice size and again dependent on the type of inkjet cartridge. A cell concentration of $7.7 \times 10^6/\text{mL}$ corresponds to one cell/droplet on an average, which can be printed with an HP26 cartridge (Parzel et al. 2009). Even though these cartridges are supposed to be able to consistently print single cells, one must take into consideration the resolution error while performing a bioprinting experiment. Resolution error can drastically affect the realistic spatial resolution of a bioprinting system. Even error in micrometers level can alter the outcome during the fabrication of tissues or biological structures with the microfabrication techniques. The main sources of resolution errors are (1) print head, (2) the drop distance (distance between the print head to the substrate where the drop will be placed), and (3) mechanical vibrations. Inkjet print heads are not designed to repeatedly deliver material to an exact point but they are designed to place the material drops on a specific area such that multiple drops create an intended spatial distribution. So, it is advised to compensate such print head-dependent errors in the design of the biological structure itself. Print heads are usually held several millimeters above their proposed target and any errors in the direction of the ink drop would be puffed up as the distance between the print head to the target increases. Finally, when creating structures on a micrometer scale, tiny vibrations from the printing system or even the movement from air conditioning system can significantly affect the final resolution. These sources of error can be reduced by fixing the print head on a stationary platform and using a moveable stage beneath the print head to create the biological structure (Binder et al. 2011).

5.4.3 Delivery matrix

The final properties of a printed biological construct are determined by the bio-material used for the matrix (Burg et al. 2010). In general, the delivery matrix is a liquid form in the print head and becomes semisolid after cross-linking

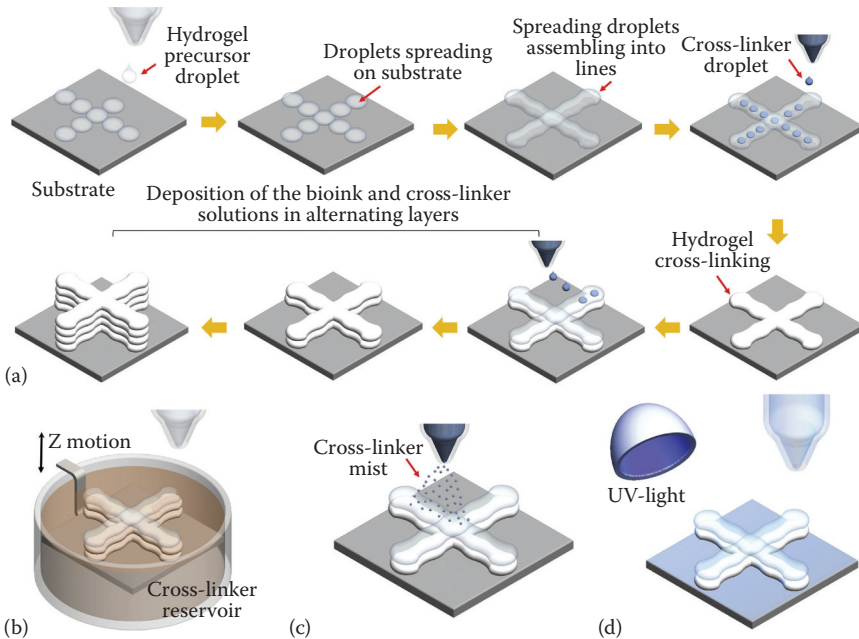


Figure 5.5 Fabrication of 3D tissue constructs by layer-by-layer deposition of bioink followed by different cross-linking strategies. (a) Droplets of a cell-laden bioink solution are deposited at targeted locations and the introduction of cross-linker solution in alternate layers. (b) Bioprinting into a reservoir filled with cross-linker. (c) Bioprinting followed by spraying the cross-linking solution. (d) Exposing each layer of the printed photocurable bioink to UV-light. (Reproduced from Gudapati, H. et al., *Biomaterials*, 102, 20–42, 2016. With permission.)

(Figure 5.5). Though a large variety of hydrogels with this property have been used for bioprinting, collagen I and alginate are the commonly used material. The role of hydrogel matrix is to provide structural stability so that a completely robust construct can be built. In addition, the selection of the matrix should be based on the biological application as it can affect the proliferation of the bioprinted cells. The matrix is expected to facilitate the nonliving and living component interactions in the printed biological structure, which is also a crucial factor in deciding the final functional characteristics of the structure (Jakab et al. 2004). Based on the required application, the matrices and the cell types may vary, for example, for the bioprinting of a simple tissue, single-cell types and matrices and multiple types of cells and materials will be required for the fabrication of a complex tissue (Binder et al. 2011).

5.4.4 Viscosity

Inkjet bioprinting exhibits constraints regarding the material viscosity. Typically, the extrusion-based techniques require shear-thinning properties to reduce the shear stress on the embedded cells to increase the cell viability (Mehta et al. 2011). Viscosity is a major determinant as only low viscous materials can be printed with inkjet bioprinters and clogging problem is prone to occur with higher viscosity materials (Hoch et al. 2013). As the bioink has low viscosity, effective, and fast, cross-linking is an important factor to be considered to facilitate the layering of the 3D-printed construct. In fact, one major drawback of inkjet bioprinting systems is nozzle clogging unless using low-viscosity hydrogels (Selimović et al. 2012, Saunders and Derby 2014, Pereira and Bártolo 2015). The viscosity of bioink often has an effect on droplet size, resolution, and reproducibility as materials with high viscosity or media suspensions with high cell density can initiate clogging due to the higher applied force to eject the drops of bioink (Lee et al. 2015). The viscosity can be modulated by mixing one or more materials or a cross-linker of interest in different ratios or addition of cells. For example, GelMA solution with a concentration of 5% is not printable whereas 10% GelMA is the commonly used concentration for tissue engineering, which shows the need of viscosity modulation (Hutson et al. 2011, Ramon-Azcon et al. 2012). A study reported that the viscosity of the prepared bioink increased significantly by adding 1.5% GelMA to the PEG solution; however, that ended up with recurrent clogging and created a hurdle for inkjet bioprinting. In contrast, PEG 10% alone showed the viscosity similar to that of regular ink whereas it was equal to that of regular ink when it was 10% PEG–peptide. Further addition of peptide solution to the concentrated PEG decreased the viscosity slightly and only a slight increase in the viscosity was reported followed by the addition of cells (Munaz et al. 2016).

5.5 Drawbacks of inkjet bioprinting

Achieving biologically significant cell density during printing is one major problem that is often encountered by users of inkjet-based bioprinting technology. Often, cell concentrations as low as 10 million cells/ml have been used for printing (Xu et al. 2005). Though a variety of materials can be printed with inkjet printing technology, viscous suspensions with high polymeric concentration or extracellular matrix (ECM) components cannot be printed directly. Therefore, cross-linkers with low viscosity are preferred to be printed onto a thick polymer solution to create a mechanically robust structure (Xu et al. 2006, Cui and Boland 2009, Chang et al. 2011). In addition, drug eluting microspheres with high concentration of rigid nano- or microparticles or calcium phosphate-like inorganic bioactive salts and high cell density inks as discussed earlier exhibit particle aggregation and clogging of the dispensing nozzle under

the applied pressure (Murphy and Atala 2014). The other reported drawbacks of inkjet bioprinting are: biological materials should be in liquid form to enable droplet formation thereafter, for a solid 3D structure, materials have to be cross-linked soon after printing or simultaneously either by photocross-linking using ultraviolet, chemical or by adjusting pH. However, the requirement for cross-linking methods often slows the process of bioprinting and involves the addition of chemicals to naturally occurring ECM materials (Khalil and Sun 2007, Murphy et al. 2013) and the consequent changes in both their chemical as well as the material properties are of major concern along with the toxicity-associated problems (Kim et al. 2003).

5.6 Challenges and future directions

Currently, advancement in the field of inkjet bioprinting is not enough to fabricate a functional human organ replacement in clinically relevant dimensions, however, it succeeded in creating multicellular microenvironments by spatial patterning. These multicellular constructs have improved the drug discovery and disease modeling in a reproducible manner (Gudapati et al. 2016). At present, many drugs available in markets are not very effective as they have been tested over a small groups of patients during clinical trials, which do not consider the genetic variations among large number of patients (Friedman et al. 2010). For instance, 97%–98% of the patients are not benefited from antihypertension drugs or statins for high cholesterol (Gudapati et al. 2016). Hence, 3D tissue models with targeted genome editing tools (Barrangou and Marraffini 2014, Hsu et al. 2014) can improve disease modeling (Hinson et al. 2015, Liu et al. 2016). Patient-customized treatment plans, drug doses, and the ability to mimic human physiology and pathology better than 2D cell cultures (Wüst et al. 2011, Billiet et al. 2012, Melchels et al. 2012) or animal models are some advantages of inkjet bioprinting (Shanks et al. 2009). Reminiscent of other advanced technologies, inkjet printing has also taken a long time to evolve to its current state. Though inkjet printing is widely accepted in laboratories and exploited commercially, a few drawbacks have been identified and put forth as potential challenges that need to be addressed immediately for its increased acceptance by industries. According to Cook et al. (2010) the extensional flow and high shear during passing of the liquid through an inkjet nozzle can significantly affect the biopharmaceuticals, specifically excipient or polymeric components. Reactive inkjet printing is an emerging separate field of study, which has particular application in drug discovery. To ensure the high-throughput screening, critical parameters such as droplet coalescence, reaction kinetics, and boundary line propagation are to be considered with great importance. At the same time the complexities associated with the laminar flow “mixing” and localized chemical kinetics also need to be considered (Smith and Morrin 2012, Castrejón-Pita et al. 2013).

The evaporation rate and volatility of the carrier fluid after printing are determinant factors of crystallization (Daly et al. 2015) and the crystal form of a pharmaceutical agent is exceedingly important (Genina et al. 2013). It is also important to consider the influence of added viscosity modifiers, excipients, and surfactants on evaporation of the carrier fluid. It is demonstrated that recrystallization behavior of active pharmaceutical ingredients varies with surface wettability and the choice of printed surface. However, Hsu et al. (2013) reported the influence of surface microstructure on recrystallization behavior of active pharmaceutical ingredients that need to be targeted with specific industrial-scale print heads. It is also reported that the chemistry of the active pharmaceutical molecule can influence the functionality of printed structure as it determines the possibility of polymorphism, ease of printing, and the sensitivity to the imparted forces (Daly et al. 2015).

Additive manufacturing has tremendous potential to create engineered tissues that could improve life for thousands of patients waiting for transplants. Akin to any other mode of tissue reconstruction, a vasculature network that allows blood and nutrients and waste flow into and out, simultaneously, is a bottleneck on a fully functional tissue regeneration by inkjet bioprinting. Due to the limited nozzle geometry, high-resolution vascular network fabrication at the single-cell level is still an unsolved problem along with restricted viscous bioink and droplet volume. Bioprinting of vasculature can be achieved by the simultaneous deposition of biocompatible and degradable support materials through strategies such as liquid buoyancy (Christensen et al. 2015). Furthermore, the currently used hydrogel biomaterials for DBB are characterized by weak mechanical strength.

Still there are a number of challenges yet to overcome toward the fabrication of functional human organ replacements (Melchels et al. 2012, Ozbolat and Yu 2013). There is a demand for upgradation in the physical characteristics of the currently available print heads to improve the long-term survivability of the construct, increased cell density/droplet, viscosity, droplet volume, and the precise positioning of droplets. The current microfabrication process for print heads restricts the flexibility in nozzle geometry. Hence, there is also a demand for micro- or nanofabrication techniques to overcome limitations associated with print head and nozzle designs. The availability of a biomimetic materials that can enhance proliferation and differentiation of cells with signaling cues and controlled degradability is another hurdle in this field (Murphy and Atala 2014).

5.7 Conclusion

Inkjet printing is a versatile technology owing to its high precision and better control over the deposition pattern. This technology has proven its prospect of enhancing the field of tissue engineering and regenerative medicine. The inkjet-based

bioprinting technology can facilitate the fabrication of functional tissue or organs. It is also capable of serving different roles in the pharmaceutical manufacturing industry from rapid drug discovery to fabrication of material for drug delivery and manufacturing of personalized medicines. Some studies in this field have already paved the way for development of more realistic approach in mimicking the native tissues. Addressing the current limitations hopefully will bring down the distance to the clinic.

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References

- Arrabito, G., C. Musumeci, V. Aiello, S. Libertino, G. Compagnini, and B. Pignataro. 2009. On the relationship between jetted inks and printed biopatterns: Molecular-thin functional microarrays of glucose oxidase. *Langmuir* 25 (11):6312–6318. doi:10.1021/la900071z.
- Barrangou, R., and L. A. Marraffini. 2014. CRISPR-Cas systems: Prokaryotes upgrade to adaptive immunity. *Molecular Cell* 54 (2):234–244. doi:10.1016/j.molcel.2014.03.011.
- Billiet, T., M. Vandenhaute, J. Schelfhout, S. Van Vlierberghe, and P. Dubruel. 2012. A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials* 33 (26):6020–6041. doi:10.1016/j.biomaterials.2012.04.050.
- Binder, K. W., A. J. Allen, J. J. Yoo, and A. Atala. 2011. DROP-on-demand inkjet bioprinting: A primer. *Gene Therapy and Regulation* 6 (1):33–49. doi:10.1142/S1568558611000258.
- Burg, T., C. A. P. Cass, R. Groff, M. Pepper, and K. J. L. Burg. 2010. Building off-the-shelf tissue-engineered composites. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* 368 (1917):1839.
- Castrejón-Pita, J. R., W. Baxter, J. Morgan, S. Temple, G. D. Martin, and I. M. Hutchings. 2013. Future, opportunities and challenges of inkjet technologies. *Atomization and Sprays* 23:571–595.
- Chang, C. C., E. D. Boland, S. K. Williams, and J. B. Hoying. 2011. Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials* 98 (1):160–170. doi:10.1002/jbm.b.31831.
- Christensen, K., C. Xu, W. Chai, Z. Zhang, J. Fu, and Y. Huang. 2015. Freeform inkjet printing of cellular structures with bifurcations. *Biotechnology and Bioengineering* 112 (5): 1047–1055. doi:10.1002/bit.25501.
- Compaan, A. M., K. Christensen, and Y. Huang. 2016. Inkjet bioprinting of 3D silk fibroin cellular constructs using sacrificial alginate. *ACS Biomaterials Science & Engineering*. doi:10.1021/acsbomaterials.6b00432.

- Cook, C. C., T. Wang, and B. Derby. 2010. Inkjet delivery of glucose oxidase. *Chemical Communications* 46 (30):5452–5454. doi:10.1039/C0CC00567C.
- Cui, X., and T. Boland. 2009. Human microvasculature fabrication using thermal inkjet printing technology. *Biomaterials* 30 (31):6221–6227. doi:10.1016/j.biomaterials.2009.07.056.
- Cui, X., T. Boland, D. D. D’Lima, and M. K. Lotz. 2012a. Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation* 6 (2):149–155.
- Cui, X., K. Breitenkamp, M. Finn, M. Lotz, and D. D. D’Lima. 2012. Direct human cartilage repair using three-dimensional bioprinting technology. *Tissue Engineering Part A* 18:1304–1312.
- Cui, X., D. Dean, Z. M. Ruggeri, and T. Boland. 2010. Cell damage evaluation of thermal inkjet printed Chinese hamster ovary cells. *Biotechnology and Bioengineering* 106 (6):963–969. doi:10.1002/bit.22762.
- Cui, X., G. Gao, and Y. Qiu. 2013. Accelerated myotube formation using bioprinting technology for biosensor applications. *Biotechnology Letters* 35 (3):315–321. doi:10.1007/s10529-012-1087-0.
- Daly, R., T. S. Harrington, G. D. Martin, and I. M. Hutchings. 2015. Inkjet printing for pharmaceuticals—A review of research and manufacturing. *International Journal of Pharmaceutics* 494 (2):554–567. doi:10.1016/j.ijpharm.2015.03.017.
- Demirci, U., and G. Montesano. 2007. Single cell epitaxy by acoustic picolitre droplets. *Lab on a Chip* 7 (9):1139–1145. doi:10.1039/B704965J.
- Miller, E. D., A. Phillippi Julie, W. Fisher Gregory, G. Campbell Phil, M. Walker Lynn, and E. Weiss Lee. 2009. Inkjet printing of growth factor concentration gradients and combinatorial arrays immobilized on biologically-relevant substrates. *Combinatorial Chemistry & High Throughput Screening* 12 (6):604–618. doi:10.2174/138620709788681907.
- Fang, Y., J. P. Frampton, S. Raghavan, R. Sabahi-Kaviani, G. Luker, C. X. Deng, and S. Takayama. 2012. Rapid generation of multiplexed cell cocultures using acoustic droplet ejection followed by aqueous two-phase exclusion patterning. *Tissue Engineering. Part C, Methods* 18 (9):647–657. doi:10.1089/ten.tec.2011.0709.
- Faulkner-Jones, A., C. Cyfe, D. J. Cornelissen, J. Gardner, K. Jason, A. Courtney, and W. Shu. 2015. Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. *Biofabrication* 7 (4):044102.
- Friedman, L. M., C. D. Furberg, and D. L. DeMets. 2010. Introduction to clinical trials. In *Fundamentals of Clinical Trials*, pp. 1–18. New York: Springer.
- Gao, G., T. Yonezawa, K. Hubbell, G. Dai, and X. Cui. 2015. Inkjet-bioprinted acrylated peptides and PEG hydrogel with human mesenchymal stem cells promote robust bone and cartilage formation with minimal printhead clogging. *Biotechnology Journal* 10 (10):1568–1577. doi:10.1002/biot.201400635.
- Gasparini, L., D. Maniglio, A. Motta, and C. Migliaresi. 2014. An electrohydrodynamic bioprinter for alginate hydrogels containing living cells. *Tissue Engineering Part C: Methods* 21 (2):123–132. doi:10.1089/ten.tec.2014.0149.
- Genina, N., D. Fors, M. Palo, J. Peltonen, and N. Sandler. 2013. Behavior of printable formulations of loperamide and caffeine on different substrates—Effect of print density in inkjet printing. *International Journal of Pharmaceutics* 453 (2):488–497. doi:10.1016/j.ijpharm.2013.06.003.
- Goldmann, T., and J. S. Gonzalez. 2000. DNA-printing: Utilization of a standard inkjet printer for the transfer of nucleic acids to solid supports. *Journal of Biochemical and Biophysical Methods* 42 (3):105–110. doi:10.1016/S0165-022X(99)00049-4.
- Gudapati, H., M. Dey, and I. Ozbolat. 2016. A comprehensive review on droplet-based bioprinting: Past, present and future. *Biomaterials* 102:20–42. doi:10.1016/j.biomaterials.2016.06.012.

- Hayati, I., A. I. Bailey, and T. F. Tadros. 1986. Mechanism of stable jet formation in electrohydrodynamic atomization. *Nature* 319 (6048):41–43.
- Hinson, J. T., A. Chopra, N. Nafissi, W. J. Polacheck, C. C. Benson, S. Swist, J. Gorham et al. 2015. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science* 349 (6251):982.
- Hoch, E., T. Hirth, G. E. M. Tovar, and K. Borchers. 2013. Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting. *Journal of Materials Chemistry B* 1 (41):5675–5685. doi:10.1039/C3TB20745E.
- Hsu, H., S. J. Toth, G. J. Simpson, L. S. Taylor, and M. T. Harris. 2013. Effect of substrates on naproxen-polyvinylpyrrolidone solid dispersions formed via the drop printing technique. *Journal of Pharmaceutical Sciences* 102 (2):638–648. doi:10.1002/jps.23397.
- Hsu, P. D., E. S. Lander, and F. Zhang. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157 (6):1262–1278. doi:10.1016/j.cell.2014.05.010.
- Hutson, C. B., J. W. Nichol, H. Aubin, H. Bae, S. Yamanlar, S. Al-Haque, S. T. Koshy, and A. Khademhosseini. 2011. Synthesis and characterization of tunable poly(ethylene glycol): Gelatin methacrylate composite hydrogels. *Tissue Engineering Part A* 17 (13–14):1713–1723. doi:10.1089/ten.tea.2010.0666.
- Ilkhanizadeh, S., A. I. Teixeira, and O. Hermanson. 2007. Inkjet printing of macromolecules on hydrogels to steer neural stem cell differentiation. *Biomaterials* 28 (27):3936–3943. doi:10.1016/j.biomaterials.2007.05.018.
- Jakab, K., A. Neagu, V. Mironov, R. R. Markwald, and G. Forgacs. 2004. Engineering biological structures of prescribed shape using self-assembling multicellular systems. *Proceedings of the National Academy of Sciences of the United States of America* 101 (9):2864–2869. doi:10.1073/pnas.0400164101.
- Jayasinghe, S. N., A. N. Qureshi, and P. A. M. Eagles. 2006. Electrohydrodynamic jet processing: An advanced electric-field-driven jetting phenomenon for processing living cells. *Small* 2 (2):216–219. doi:10.1002/smll.200500291.
- Jayasinghe, S. N., and A. Townsend-Nicholson. 2006. Stable electric-field driven cone-jetting of concentrated biosuspensions. *Lab on a Chip* 6 (8):1086–1090. doi:10.1039/B606508M.
- Kesti, M., P. Fisch, M. Pensalfini, E. Mazza, and M. Zenobi-Wong. 2016. Guidelines for standardization of bioprinting: A systematic study of process parameters and their effect on bioprinted structures. *BioNanoMaterials* 17 (3–4):193–204.
- Khalil, S., and W. Sun. 2007. Biopolymer deposition for freeform fabrication of hydrogel tissue constructs. *Materials Science and Engineering: C* 27 (3):469–478. doi:10.1016/j.msec.2006.05.023.
- Khanarian, N. T., J. Jiang, L. Q. Wan, V. C. Mow, and H. H. Lu. 2011. A hydrogel-mineral composite scaffold for osteochondral interface tissue engineering. *Tissue Engineering Part A* 18 (5–6):533–545. doi:10.1089/ten.tea.2011.0279.
- Kim, H. S., D. Y. Lee, J. H. Park, J. H. Kim, J. H. Hwang, and H. I. Jung. 2007. Optimization of electrohydrodynamic writing technique to print collagen. *Experimental Techniques* 31 (4):15–19. doi:10.1111/j.1747-1567.2007.00154.x.
- Kim, T. K., B. Sharma, C. G. Williams, M. A. Ruffner, A. Malik, E. G. McFarland, and J. H. Elisseeff. 2003. Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage. *Osteoarthritis and Cartilage* 11 (9):653–664. doi:10.1016/S1063-4584(03)00120-1.
- Klebe, R. J. 1988. Cytoscribing: A method for micropositioning cells and the construction of two- and three-dimensional synthetic tissues. *Experimental Cell Research* 179 (2):362–373.
- Lee, V. K., A. Dias, M. S. Ozturk, K. Chen, B. Tricomi, D. T. Corr, X. Intes, and G. Dai. 2015. 3D bioprinting and 3D imaging for stem cell engineering. In *Bioprinting in Regenerative Medicine*, K. Turksen (Ed.), pp. 33–66. Cham, Switzerland: Springer International Publishing.

- Liu, J., C. Gao, W. Chen, W. Ma, X. Li, Y. Shi, H. Zhang et al. 2016. CRISPR/Cas9 facilitates investigation of neural circuit disease using human iPSCs: Mechanism of epilepsy caused by an SCN1A loss-of-function mutation. *Translational Psychiatry* 6:e703. doi:10.1038/tp.2015.203.
- Mehta, G. K., S. Kondaveeti, and A. K. Siddhanta. 2011. Facile synthesis of agarose-l-phenylalanine ester hydrogels. *Polymer Chemistry* 2 (10):2334–2340. doi:10.1039/C1PY00250C.
- Melchels, F. P. W., M. A. N. Domingos, T. J. Klein, J. Malda, P. J. Bartolo, and D. W. Huttmacher. 2012. Additive manufacturing of tissues and organs. *Progress in Polymer Science* 37:1079–1104. doi:10.1016/j.progpolymsci.2011.11.007.
- Miller, E. D., G. W. Fisher, L. E. Weiss, L. M. Walker, and P. G. Campbell. 2006. Dose-dependent cell growth in response to concentration modulated patterns of FGF-2 printed on fibrin. *Biomaterials* 27 (10):2213–2221. doi:10.1016/j.biomaterials.2005.10.021.
- Mohebi, M. M., and J. R. G. Evans. 2002. A drop-on-demand ink-jet printer for combinatorial libraries and functionally graded ceramics. *Journal of Combinatorial Chemistry* 4 (4):267–274. doi:10.1021/cc010075e.
- Munaz, A., R. K. Vadivelu, J. S. John, M. Barton, H. Kamble, and N. T. Nguyen. 2016. Three-dimensional printing of biological matters. *Journal of Science: Advanced Materials and Devices* 1 (1):1–17. doi:10.1016/j.jsamd.2016.04.001.
- Murphy, S. V., and A. Atala. 2014. 3D bioprinting of tissues and organs. *Nature Biotechnology* 32 (8):773–785. doi:10.1038/nbt.2958.
- Murphy, S. V., A. Skardal, and A. Atala. 2013. Evaluation of hydrogels for bio-printing applications. *Journal of Biomedical Materials Research Part A* 101A (1):272–284. doi:10.1002/jbm.a.34326.
- Nakamura, M., A. Kobayashi, F. Takagi, A. Watanabe, Y. Hiruma, K. Ohuchi, Y. Iwasaki, M. Horie, I. Morita, and S. Takatani. 2006. Biocompatible inkjet printing technique for designed seeding of individual living cells. *Tissue Engineering* 11 (11–12):1658–1666. doi:10.1089/ten.2005.11.1658.
- Nishiyama, Y., M. Nakamura, C. Henmi, K. Yamaguchi, S. Mochizuki, H. Nakagawa, and K. Takiura. 2008. Development of a three-dimensional bioprinter: Construction of cell supporting structures using hydrogel and state-of-the-art inkjet technology. *Journal of Biomechanical Engineering* 131 (3):035001. doi:10.1115/1.3002759.
- Okamoto, T., T. Suzuki, and N. Yamamoto. 2000. Microarray fabrication with covalent attachment of DNA using bubble jet technology. *Nature Biotechnology* 18 (4):438–441.
- Onses, M. S., E. Souto, P. M. Ferreira, A. G. Alleyne, and J. A. Rogers. 2015. Mechanisms, capabilities, and applications of high-resolution electrohydrodynamic jet printing. *Small* 11 (34):4237–4266. doi:10.1002/sml.201500593.
- Ozolat, I. T., and Y. Yu. 2013. Bioprinting toward organ fabrication: Challenges and future trends. *IEEE Transactions on Biomedical Engineering* 60 (3):691–699. doi:10.1109/TBME.2013.2243912.
- Parzel, C. A., M. E. Pepper, T. Burg, R. E. Groff, and K. J. L. Burg. 2009. EDTA enhances high-throughput two-dimensional bioprinting by inhibiting salt scaling and cell aggregation at the nozzle surface. *Journal of Tissue Engineering and Regenerative Medicine* 3 (4):260–268. doi:10.1002/term.162.
- Pataky, K., T. Braschler, A. Negro, P. Renaud, M. P. Lutolf, and J. Brugger. 2012. Microdrop printing of hydrogel bioinks into 3D tissue-like geometries. *Advanced Materials* 24 (3):391–396. doi:10.1002/adma.201102800.
- Pereira, R. F., and P. J. Bártolo. 2015. 3D bioprinting of photocrosslinkable hydrogel constructs. *Journal of Applied Polymer Science* 132 (48). doi:10.1002/app.42458.
- Poellmann, M. J., K. L. Barton, S. Mishra, and A. J. Wagoner Johnson. 2011. Patterned hydrogel substrates for cell culture with electrohydrodynamic jet printing. *Macromolecular Bioscience* 11 (9):1164–1168. doi:10.1002/mabi.201100004.

- Ramon-Azcon, J., S. Ahadian, R. Obregon, G. Camci-Unal, S. Ostrovidov, V. Hosseini, H. Kaji et al. 2012. Gelatin methacrylate as a promising hydrogel for 3D microscale organization and proliferation of dielectrophoretically patterned cells. *Lab on a Chip* 12 (16):2959–2969. doi:10.1039/C2LC40213K.
- Rayleigh, L. 1878. On the instability of jets. *Proceedings of the London Mathematical Society* s1–10 (1):4–13. doi:10.1112/plms/s1-10.1.4.
- Saunders, R. E., and B. Derby. 2014. Inkjet printing biomaterials for tissue engineering: Bioprinting. *International Materials Reviews* 59 (8):430–448. doi:10.1179/1743280414Y.0000000040.
- Saunders, R. E., J. E. Gough, and B. Derby. 2008. Delivery of human fibroblast cells by piezo-electric drop-on-demand inkjet printing. *Biomaterials* 29 (2):193–203. doi:10.1016/j.biomaterials.2007.09.032.
- Selimović, Š., J. Oh, H. Bae, M. Dokmeci, and A. Khademhosseini. 2012. Microscale strategies for generating cell-encapsulating hydrogels. *Polymers* 4 (3):1554–1554.
- Shanks, N., R. Greek, and J. Greek. 2009. Are animal models predictive for humans? *Philosophy, Ethics, and Humanities in Medicine: PEHM* 4:2–2. doi:10.1186/1747-5341-4-2.
- Sirringhaus, H., T. Kawase, R. H. Friend, T. Shimoda, M. Inbasekaran, W. Wu, and E. P. Woo. 2000. High-resolution inkjet printing of all-polymer transistor circuits. *Science* 290 (5499):2123.
- Skardal, A., D. Mack, E. Kapetanovic, A. Atala, J. D. Jackson, J. Yoo, and S. Soker. 2012. Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. *Stem Cells Translational Medicine* 1 (11):792–802. doi:10.5966/sctm.2012-0088.
- Smith, P. J., and A. Morrin. 2012. Reactive inkjet printing. *Journal of Materials Chemistry* 22 (22):10965–10970. doi:10.1039/C2JM30649B.
- Spalazzi, J. P., E. Dagher, S. B. Doty, X. E. Guo, S. A. Rodeo, and H. H. Lu. 2008. In vivo evaluation of a multiphased scaffold designed for orthopaedic interface tissue engineering and soft tissue-to-bone integration. *Journal of Biomedical Materials Research Part A* 86A (1):1–12. doi:10.1002/jbm.a.32073.
- Tasoglu, S., and U. Demirci. 2013. Bioprinting for stem cell research. *Trends in Biotechnology* 31 (1):10–19. doi:10.1016/j.tibtech.2012.10.005.
- Technote, MicroFab. 1999. Background on ink-jet technology. In MicroFab technote 99-01. Plano, TX: Microfab Technologies.
- Tekin, E., P. J. Smith, and U. S. Schubert. 2008. Inkjet printing as a deposition and patterning tool for polymers and inorganic particles. *Soft Matter* 4 (4):703–713. doi:10.1039/B711984D.
- Tibbitt, M. W., and K. S. Anseth. 2009. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnology and Bioengineering* 103 (4):655–663. doi:10.1002/bit.22361.
- Wilson, W. C., and T. Boland. 2003. Cell and organ printing 1: Protein and cell printers. *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology* 272A (2):491–496. doi:10.1002/ar.a.10057.
- Workman, V. L., L. B. Tezera, P. T. Elkington, and S. N. Jayasinghe. 2014. Controlled generation of microspheres incorporating extracellular matrix fibrils for three-dimensional cell culture. *Advanced Functional Materials* 24 (18):2648–2657. doi:10.1002/adfm.201303891.
- Wüst, S., R. Müller, and S. Hofmann. 2011. Controlled positioning of cells in biomaterials—Approaches towards 3D tissue printing. *Journal of Functional Biomaterials* 2 (3):119–154. doi:10.3390/jfb2030119.
- Xu, C., W. Chai, Y. Huang, and R. R. Markwald. 2012. Scaffold-free inkjet printing of three-dimensional zigzag cellular tubes. *Biotechnology and Bioengineering* 109 (12):3152–3160. doi:10.1002/bit.24591.
- Xu, T., K. W. Binder, M. Z. Albanna, D. Dice, W. Zhao, J. J. Yoo, and A. Atala. 2013a. Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications. *Biofabrication* 5 (1):015001.

- Xu, T., C. A. Gregory, P. Molnar, X. Cui, S. Jalota, S. B. Bhaduri, and T. Boland. 2006. Viability and electrophysiology of neural cell structures generated by the inkjet printing method. *Biomaterials* 27 (19):3580–3588. doi:10.1016/j.biomaterials.2006.01.048.
- Xu, T., J. Jin, C. Gregory, J. J. Hickman, and T. Boland. 2005. Inkjet printing of viable mammalian cells. *Biomaterials* 26 (1):93–99. doi:10.1016/j.biomaterials.2004.04.011.
- Xu, T., H. Kincaid, A. Atala, and J. J. Yoo. 2008. High-throughput production of single-cell microparticles using an inkjet printing technology. *Journal of Manufacturing Science and Engineering* 130 (2):021017. doi:10.1115/1.2903064.
- Xu, T., W. Zhao, J. M. Zhu, M. Z. Albanna, J. J. Yoo, and A. Atala. 2013b. Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology. *Biomaterials* 34 (1):130–139. doi:10.1016/j.biomaterials.2012.09.035.
- Yamaguchi, S., A. Ueno, Y. Akiyama, and K. Morishima. 2012. Cell patterning through inkjet printing of one cell per droplet. *Biofabrication* 4 (4):045005.

Chapter 6 Rapid prototyping of soft bioactuators

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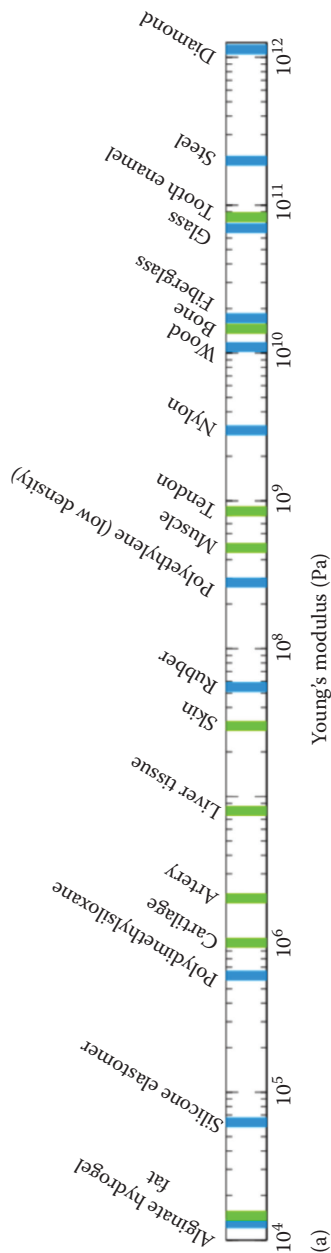
6.1 Background: Bioinspiration in tissue engineering and robotic actuators

The driving principle behind man-made robots is force actuation leading to a form of directed movement or locomotion. Natural systems can motivate the design and development of robots that replicate or enhance many basic locomotive strategies—such as climbing, crawling,¹ walking,² jumping,^{3,4} or swimming^{5–9}—with novel solutions. Biological soft robotics derives inspiration and design principles from organic systems to facilitate engineering approaches to challenges that have historically plagued conventional robotic actuators. Traditional hard skeletons (made of high stiffness metals or plastics) and electromagnetic actuators can

result in rigid bioactuators that exhibit few degrees of freedom (DOF), low complexity, difficulty in grasping actions, and aggressive collisions with living tissues. Moreover, they rarely present multifunctionality, versatility, or adaptability.¹⁰

Conversely, soft bioactuators (typically composed of gels, polymers, and fluids, sometimes with the addition of biological materials) must not only be functional in a research laboratory but also effective in situations where they may be called on to move over unstable terrain while carrying heavy loads of sensors, imagers, or samplers. These devices would also ideally be capable of untethered as well as directional locomotion, elastic deformation or stiffness modulation, efficient energy storage, and robust motion control, to be both effective and useful. Finally, these continuum robots must be environmentally safe and sufficiently low cost such that they can be abandoned if damaged or polluted.¹¹ Soft biorobotic manipulators with high power-to-weight ratios generally have more DOF and are more compliant than their rigid counterparts, and can manipulate fragile and unknown objects via a simple control algorithm. The lightweight and flexible polymers, hydrogels, and elastomers used to form soft robots have lower stiffness (moduli of 10^4 – 10^9 Pa) that corresponds to properties of biological matter with which they might interact (Figure 6.1a). Due to recent manufacturing advancements, they can be rapidly produced with high spatial control and a range of properties in three dimensions.^{3,12–15}

Beyond structures, soft biorobotic systems require an actuating source and fuel supply. It therefore follows that living biological materials (or relevant mimics thereof) would inspire and comprise a large portion of demonstrated bioactuators.^{15,16} Beyond simple biomimicry, the field of *biodesign* incorporates living organisms into artificial or manmade systems.¹⁷ The addition of living biological actuator sources (e.g., muscle tissues) can increase the efficiency and responsiveness of soft actuators, as many of these living components have evolved with efficient standard processes for force production, energy consumption, or net movement.^{18–21} The world of biology is full of intricate systems designed to solve extremely complex locomotive and manipulative tasks with high efficiency at a wide variety of scales. Depending on the ecosystem, there are numerous methods of potential locomotion among diverse structures and species, including both plants and animals.²² Considering the breadth of methods of propulsion, it is perhaps unsurprising that there are also many ways for these robots to generate a range of locomotive forces, from molecular (e.g., motor proteins; 10^{-9} m) to cellular (e.g., individual microorganisms or cells; 10^{-6} m) to tissue (e.g., muscles or cell clusters; 10^{-3} m) length scales.²¹ In addition to locomotors, there are also biorobots that imitate peristalsis to act as pumps or valves, transport cargo, actuate a joint, sense a signal, or perform as microgrippers, rotors, mixers, or manipulators to achieve other tasks.^{23–26} It is apparent that the development of soft bioactuators necessitates the intersection and integration of advancements in diverse fields such as nanotechnology, tissue engineering (TE), and developmental biology.²⁷ In this chapter, we discuss the use of rapid prototyping technologies to achieve that end.



(a)

Figure 6.1 Rapid prototyping methods. (a) Range of tensile elastic moduli of biological and synthetic materials. Soft bioactuators mimic the material properties of biological tissues. (Reprinted by permission from Macmillan Publishers Ltd. *Nature*, Rus, D. and Tolley, M.T., 2015, copyright 2015.)

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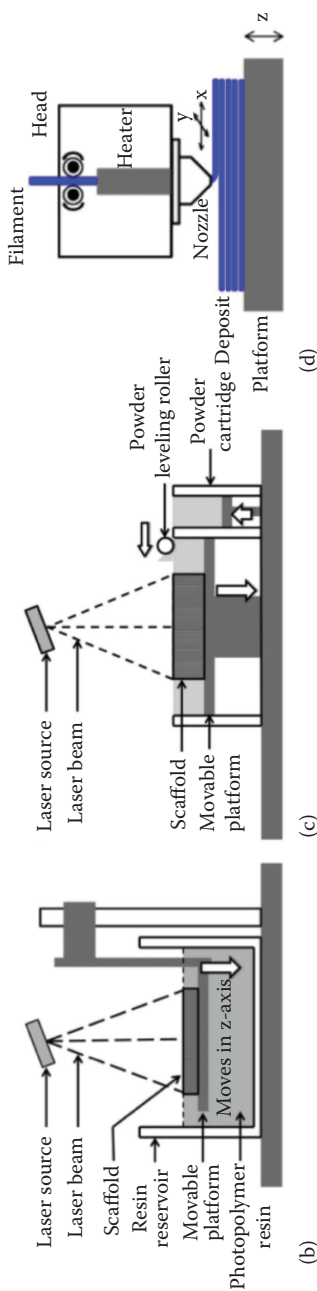


Figure 6.1 (Continued) Rapid prototyping methods. (b) Stereolithography (SL) is a maskless solid free-form method that requires a photosensitive resin and a UV light source. The apparatus (SLA) contains a platform that moves vertically. This technique traces the laser or projected image to photopolymerize a liquid resin in a layer-by-layer fashion. (Reprinted from Wu, G.H. and Hsu, S.H., *J. Med. Biol. Eng.*, 35, 285–292, 2015. With permission.) (c) Selective laser sintering (SLS) is the laser-based fusing of thermoplastics or metal powders to create a desired 3D structure. When a laser hits the first layer of powder on the stage, the particles fuse. A new powdered layer is deposited on the first layer and exposed to the laser again. After the fabrication is complete, the unsintered powder is removed. (Reprinted from Wu, G.H. and Hsu, S.H., *J. Med. Biol. Eng.*, 35, 285–292, 2015. With permission.) (d) Fused deposition modeling (FDM) is a solid-based system that utilizes extrusion. The nozzles (which can dispense different materials) extrude a filament-shaped material on the stage to pattern the first layer; then, the platform lowers and the next layer subsequently forms on the first. As the nozzle dispenses the material at high temperature, all the layers bind well without additional treatment. (Reprinted from *J. Mater. Process Technol.*, 209, Ahn, D. et al., Representation of surface roughness in fused deposition modeling, 5593–5600, Copyright 2009, with permission from Elsevier.) (Continued)

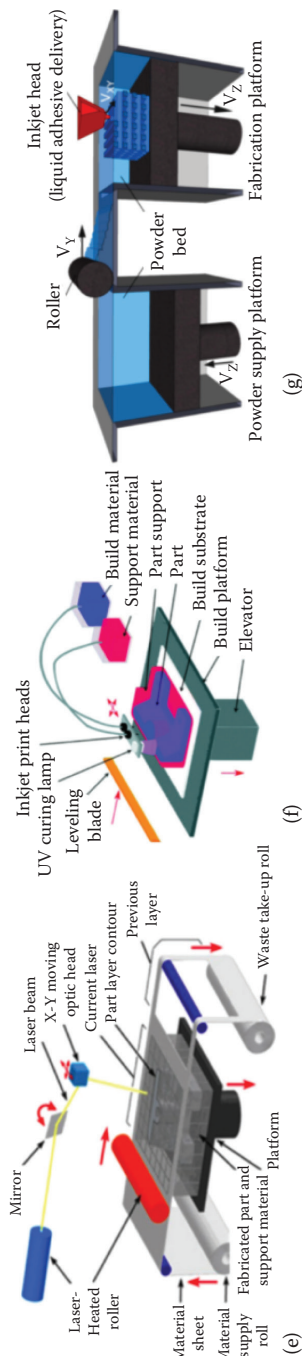


Figure 6.1 (Continued) Rapid prototyping methods. (e) With laminated object manufacturing (LOM), a single layered material, such as paper, plastic, or metal, is adhered to the platform. Then, a laser beam sketches the outline by cutting and removing the excessive material. The platform moves down by thickness of a single layer and another sheet of material is adhered on the first layer. The process is repeated until the final layer is patterned. LOM is economical and fast but not effective for fabricating geometrically complex designs. (Reprinted from *J. Mater. Process Technol.*, 212, Ahn, D. et al., Quantification of surface roughness of parts processed by laminated object manufacturing, 339–346, Copyright 2012, with permission from Elsevier.) (f) MultiJet Modeling (MJM) combines different RP techniques. A base thermoplastic polymer material is dispensed from an array of nozzles moving in the X-Y plane. The array draws the desired shape with the polymer on the platform, which then lowers relative to the array before the next layer is formed. (Bhattacharjee, N. et al., *Lab Chip*, 16, 1720–1742, 2016. Reproduced by permission of The Royal Society of Chemistry.) (g) Three-dimensional printing (3DP) can employ an inkjet type processing method. There are two adjacent chambers in this system. A feed roller on top of the material chamber spreads a powdered plaster material evenly on a platform moving vertically in the build chamber. A cartilage hanging above the build chamber moves over the surface to dispense a binding material. The build chamber moves down by one layer thickness, the process is repeated, and excess material is removed. Compared to other RP technologies, 3DP is fast and starting material is inexpensive. (Reprinted from *Biomaterials*, 33, Billiet, T. et al., A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering, 6020–6041, Copyright 2012, with permission from Elsevier.)

6.2 Rapid prototyping techniques and applications

The malleable nature, micro- to macro-scales, and potential for intricate composition and structure of bioactuators suggest that researchers will require a different manufacturing approach than can generally be employed for rigid robots. Rapid prototyping (RP) refers to a group of techniques that collect digital information to robotically fabricate physical 3D polymers, metals, and ceramic solids.^{28–30} These 3D structures are dictated by a computer-aided design (CAD) model of the desired part, which can be built from scratch or derived from medical images to print patient-specific structures. Although various techniques exist (Figure 6.1b–g), all methods of RP adapt a similar fundamental approach: a CAD model is converted into a standard tessellated language (STL) file; a computer program then receives the information and slices the 3D model; and finally, these cross sections are sequentially layered using additive manufacturing technologies that employ extrusion, melting, jetting, or photopolymerization to create a final structure. All RP techniques have short fabrication times, low costs, minimal postprocessing and waste, and variable material choices and properties, with resolutions that extend from micron to centimeter scale.^{18,31–33}

CAD not only serves as the input for an important method by which soft robotic actuators or patient-specific scaffolds can be fabricated but also provides a substrate for virtual testing and development. Due to both the increasing ubiquity of (and immense improvements in) computational power, simulation tools can now be used to calculate kinematic, dynamic, and finite-element analysis-based responses of a prototype and visualize the results in an interactive, 3D virtual environment. The ability to model prototypes realistically and accurately while validating preliminary prototype results has become integral in nearly all facets of engineering design, including bioinspired robotics.³⁴ Finally, CAD libraries can also be utilized for input on material selection and design optimization.³⁵

RP allows for the fabrication of complex and multilayered 3D structures and geometries that cannot be achieved using conventional multistep processes such as mask-based soft lithography and was therefore introduced to TE to overcome limitations of conventional fabrication techniques.^{24,36,37} For example, some RP techniques could allow for printing of spatially controlled growth factors or the use of multiple nozzles loaded with different biomaterials to create more advanced tissue structures composed of diverse matrix components.^{38–40} Most important, stereolithography (SL) enables the construction of micro- or mesoscale tissue structures with desired shapes and physical properties,⁴¹ and provides the user with a wide range of synthetic and natural material options (e.g., hydrogels that can encapsulate living cells^{42,43}) that offer greater versatility and compatibility than PDMS or metallic structures. Furthermore, through the specification of light intensity, irradiation time, and chemical makeup of the liquid resin, the mechanical properties of a printed part can be precisely regulated. The ability to fine-tune

these features, including strength and porosity, can have implications for cells that are sensitive to the stiffness, topography, and geometry of their microenvironment.^{42,44–47} Moreover, CAD allows for the production of advanced designs that closely resemble the physical morphology, orientation, or finer details of native tissue; interconnected pores, complex surface topography, and internal structures can be easily reconstructed into rapidly manufactured scaffolds.^{48–50}

Due to increasing automation speeds as well as high throughput and iterative capabilities that allow for design optimization, rapid prototyping technologies are especially useful for the production of soft bioactuators in an inexpensive and mass-producible manner.^{10,30,51} These techniques can be utilized to construct temporary or sacrificial shape-specific molds, or to print the bodies of flexible bio-actuating devices themselves.¹⁹ In the case of the latter, high-yield RP allows for manufacturing of complex structures in a single-step process, with the possibility of shape variations or heterogeneous properties.¹⁸

Each RP technique requires different materials in a specific form. The selected material thus needs to be compatible with the fabrication method as well as the intended application. In addition, the specific architecture of the scaffold depends on the type of RP technique. For example, selective laser sintering (SLS) of powders is not suitable for building porous structures or smooth surfaces; on the other hand, extrusion-based fused deposition modeling (FDM) produces thermoplastic parts with smooth surfaces that need further modification to ensure cell adhesion. Soft actuators necessitate a flexible biodegradable or biocompatible polymer.^{18,40,51} Therefore, it is important to consider material properties and the design of the scaffold, whether the user desires to successfully regenerate a tissue or build a functional bioactuator. The user's choice of RP fabrication technique, materials, and biological actuating source (if applicable), should be entirely context- and application-dependent.

6.3 Nonliving bioactuators

In many cases, synthetic RP devices have been developed to mimic the agonist–antagonist style of actuation that characterizes living tissues (Figure 6.2a–d). While they do exhibit many favorable characteristics (such as greater DOF and flexibility) compared to rigid actuators and are not subject to the sensitivity or strict environmental conditions necessitated by metabolically active cells, nonliving soft bioactuators can be at a loss when compared to the volumetric efficiency of native muscle, or the controllability of rigid actuators. Moreover, these systems often require an external power source, which adds extraneous weight that further increases energy requirements. This additional hardware, however, can contribute to significantly improved power density for synthetic actuators. The movement of nonliving soft devices can be controlled with fluidic elastomer actuators (FEAs),

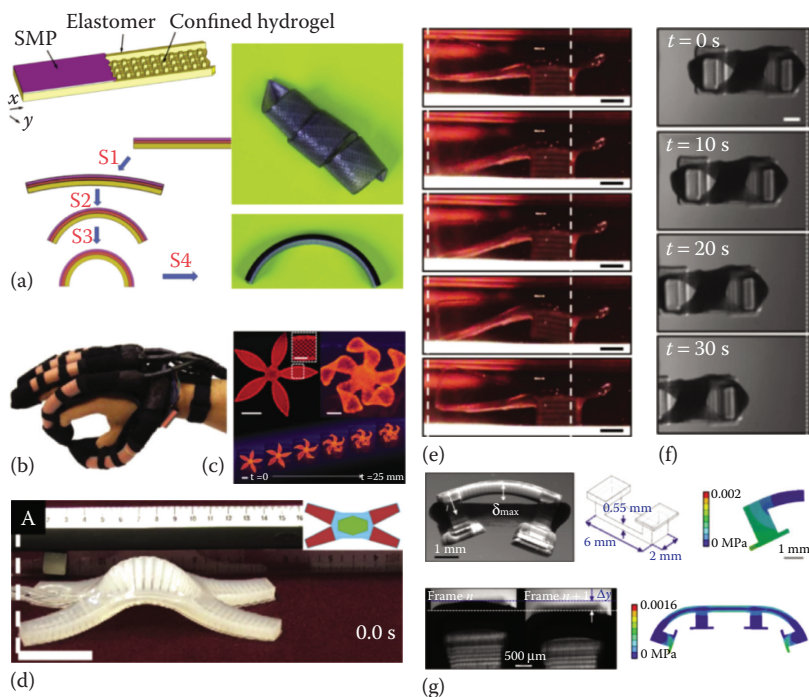


Figure 6.2 Soft bioactuators. Examples of soft bioactuators fabricated with rapid prototyping methods are demonstrated in both nonliving (a–d) and living (e–g) systems. (a) The combination of hydrogels and SMPs allowed for a two-way actuator that could reversibly bend or coil. (Adapted from Mao, Y. et al., *Sci. Rep.*, 6, 24761, 2016. With permission.) (b) A hydraulic-powered soft robotic glove fabricated from a 3D-printed mold was able to demonstrate precise grasping. (Reprinted from *Rob. Auton. Syst.*, 73, Polygerinos, P. et al., Soft robotic glove for combined assistance and at-home rehabilitation, 135–143, Copyright 2015, with permission from Elsevier.) (c) The complex morphological transformation of a flower structure was made possible with 4D bioprinting. (Reprinted by permission from Macmillan Publishers Ltd. *Nat. Mater.*, Gladman, A.S. et al., 2016, copyright 2016.) (d) An elastomeric crawling soft robot was powered by pneumatic pressure. (Reprinted from Shepherd, R.F. et al., *Proc. Natl. Acad. Sci. USA*, 108, 20400–20403, 2011. With permission.) (e) A 3D-printed biohybrid cantilever was powered by the spontaneous contraction of cardiac muscle cells. (Reprinted by permission from Macmillan Publishers Ltd. *Sci. Rep.*, Chan, V. et al., 2012, copyright 2012.) (f) An electrically powered biobot was fabricated from a hydrogel skeleton made with a stereolithographic 3D printer and a combination of skeletal muscle cells and ECM proteins. (Reprinted from Cvetkovic, C. et al., *Proc. Natl. Acad. Sci. USA*, 111, 10125–10130, 2014. With permission.) (g) An optically powered and exercised skeletal muscle biobot demonstrated directionality and control. (Reprinted from Raman, R. et al., *Nat. Protoc.*, 12, 519–533, 2017. With permission.)

(which utilize pneumatic or hydraulic pressure), variable-length tendons (such as tension cables or shape-memory materials), or electroactive polymers (EAPs), (which can be ionic or electronic).^{18,52} Considering the advantages, it is not surprising that RP fabrication methods also permit low production costs and printing times for soft bioactuators.^{19,53} 3D-printed soft actuator materials, utilized stimuli, speeds, and pros and cons are reviewed in References.^{1,51,52}

6.3.1 Fluidic elastomer actuators

FEAs are a frequently utilized actuation method that relies on pressurized fluid or compressed air for controlled structural deformation. Design or geometric asymmetry can allow for net movement on fluid inflation. Pneumatic systems (such as McKibben actuators⁵⁴ or artificial muscles) generally consist of both extensible and inextensible but flexible layers, broken up into a series of internal chambers or channels. On pressurization, the more inflexible shell constrains the material to increase its diameter, shorten, and exhibit greater rigidity or stiffness—that is, to contract like muscle.^{52,55} These versatile soft robots can be modeled after worms,⁵⁶ octopuses,⁵⁷ flat muscles,⁵⁸ and novel multilimbed organisms.⁵⁹ Recent advances have resulted in the development of soft, miniaturized pneumatic hardware that allows the robots to behave somewhat autonomously, such as crawling through tightly confined spaces—which would be impossible to navigate with rigid or tethered robots.^{60,61} Pneumatic actuators are lightweight, robust, and easily controlled; moreover, they can operate in wider temperature ranges than muscle *in vivo*.⁶² However, some pneumatic systems still lack robustness, reliability, and overall control.⁶³

Yang et al. created a variable stiffness robotic finger that exhibited a change in elastic modulus of the 3D-printed shape memory polymer (SMP) (Section 6.3.2) skeleton with temperature. Heating of selective regions within the pneumatic actuator caused bending of the substrate and could be modified to achieve gripping or grasping.⁶⁴ Bartlett et al. 3D printed a multimaterial robot that exhibited a wide stiffness gradient within its body. The jumping robot was powered by both a combustion reaction within the body's chamber as well as inflation of its pneumatic legs.⁴ Recently, Wang et al. directly 3D printed an air-driven soft robot, with integrated curvature sensors, capable of gripping. It has been hypothesized that the use of soft grippers could increase safety and decrease scar formation in surgical applications.^{55,65}

Some work has moved away from the use of air as its medium and instead incorporates denser fluids in the creation of hydraulic-powered soft robots.⁶⁶ MacCurdy et al. used a five-head printer to fabricate a bellows actuator of solid polymers and liquid material simultaneously. The legs were actuated by pumping fluid throughout the bellows of the hexapod robot's body.⁶⁷ Using propulsion principles seen in the octopus, Fischer et al. created a hydraulic underwater actuator by using

FDM to fabricate a flexible thermoplastic material.⁶⁸ Hydraulic power increases the ceiling frequency of actuation and provides higher forces and durations of actuation than pneumatic pressure. However, pneumatic actuation is more environmentally benign and exhibits less weight.^{19,69}

6.3.2 Variable-length tendon actuators and *smart* materials

Variable-length tendon actuators that conform or morph their properties can take the form of shape memory alloy (SMA) actuators and lightweight metals with highly tunable mechanical properties depending on the specific alloy used, or tension cables, which require an external conventional motor. Advantages of SMAs include minimal weight and bidirectional scaling. As shown in tentacular soft robots,⁶⁰ application of a thermoelectric stimulus to composite SMAs can result in directed movement with high force and large DOF during a temperature-induced phase transformation. However, the phase transition is relatively slow and lacking in high precision, and researchers lack a targeted method for heating the wires.^{55,70}

The materials used in soft robotics must contain an additional level of complexity that allows them to be stable along a wide range of environmental conditions but undergo drastic conformational changes on variation of this environmental stimulus around a given critical point.⁷¹ A common category of bioactuator utilizes rapidly prototyped or printed *smart* materials that can physically react to dynamic stimuli. Though response time and control mechanisms vary by stimulus, these materials can controllably and reversibly respond to changes in pH, light, pressure, moisture, temperature, ionic gradient, and electric or magnetic fields by altering one or more physical properties, such as contraction or expansion of shape—much like an organism might do when subjected to varying ecological conditions.^{71–75}

Combining smart materials with rapid prototyping can have interesting outcomes for soft biorobotics. For example, a composite biomimetic actuating system could contain SMAs embedded in soft 3D-printed materials for greater control. These smart materials can provide actuation power and structural support, lending soft bioactuators' increased flexibility and dexterity. Walters et al. prototyped a tentacle-like elastomer fabricated by 3D printing and actuated by inserted SMAs.⁷⁶ Gui et al. manufactured a tripedal soft robot modeled after a spider. Forward locomotion was powered by an SMA *muscle* (a metal fiber) directly printed into a soft 3D photopolymer adhesive structure.¹¹ Drawing inspiration from the deformation of crawling and climbing insects, Umedachi et al. designed electrically powered, SMA- or electric tendon-actuated *softworms*. These multi-limbed actuators contained a rubber body fabricated from multimaterial 3D printing. SMAs provided structural support and actuation; variable friction control allowed net forward crawling, as well as control of speed and steering.^{1,77,78} The combination of 3D

fabrication with controllable spatiotemporal properties is sometimes referred to as *four-dimensional (4D) printing*.^{70,72}

SMPs can recover their original conformation from a temporary stimulus-induced change in shape. Compared to SMAs, these softer materials are cheaper and have a larger range of tunable properties (mechanical, thermal, or optical), and are more similar to native muscle.^{72,79,80} Most important, they do not require the extensive current supply or activation heat of SMAs.¹⁸ Bodaghi et al. printed SMPs into various arrangements of flexible beams in planar and tubular arrangements. A stress anisotropy resulted in expansion and shrinking of the polymer bioactuator on thermomechanical stimulus.⁸¹ Mao et al. designed a 3D-printed arrangement of two different materials. Two-way actuation was achieved as the system could reversibly switch between twofold stable configurations in response to temperature (SMPs) or water absorption (hydrogel) stimuli.⁸² Wu et al. controlled the bending, folding, and opening of 2D substrates by adjusting the SMP fiber-volume fraction within a 3D-printed composite to mimic insect, helix, and hook designs.⁸³

Hydrogels are capable of swelling on water absorption and can be considered *smart* materials for 4D printing. Bakarich et al. developed a thermally stimulated actuator with large strain by printing an ionic covalent entanglement hydrogel (PNIPAAm) with high toughness. The transition of the material at a critical temperature caused a decrease in water content and change in volume.⁷⁴ Sydney Gladman et al. printed a patterned hydrogel composite out of a soft polyacrylamide matrix with embedded stiff cellulose fibrils. The multimaterial system mimicked a plant cell wall composition and produced a controlled curvature due to anisotropic swelling when immersed in water.⁷² Zolfagharian et al. demonstrated a photo-thermal-responsive bioactuator, additively manufactured from an extrusion-based chitosan hydrogel, with remote control over folding.⁸⁴ Zhu et al. developed an optical 3D-printing technology to fabricate an artificial PEG–hydrogel microfish with magnetic guidance.⁸⁵

6.3.3 Electroactive polymer actuators

When subjected to an electric field stimulus, EAPs are capable of changes in overall shape, resulting in strain and therefore actuation. These materials have been utilized for a range of applications, including the development of electrically active soft bioactuators.^{55,86} Asaka et al. presented a thorough review of EAP materials and biomedical applications in *Current Status of Applications and Markets of Soft Actuators*.⁸⁷ Dielectric elastomer actuators (DEAs) are capable of thickness contraction and area expansion under high voltage. They can demonstrate high strain (200%), elasticity, efficiency, and energy density. Rossiter et al. fabricated a DEA using a combination of 3D-printing techniques with soft and rigid materials. A simple antagonistic structure composed of two membranes was proposed as a prototype for soft robotics.⁸⁸ Recently, Cai et al. developed an

acrylic DEA to robotically mimic native facial muscles using both FDM for the frame and a multimaterial 3D printer for the dielectric film,⁸⁹ and Nguyen et al. fabricated a scalable DEA hexapod robot with controllable directional locomotion, rotation, and turning.⁹⁰

Ionic polymer-metal composites (IPMCs) require low actuating voltages to change shape or bend and are therefore promising for soft robotics. They must operate in wet conditions (amenable to swimming bioactuators) and thus require some protection in air.^{14,19,52,76} While these compliant materials have been developed as intelligent artificial muscles, only few groups have constructed soft EAP bioactuators with rapid prototyping technologies.⁸⁶ Carrico et al. demonstrated a novel method for printing soft IMPC structures using fused filament additive manufacturing. A polymer was printed in a layer-by-layer fashion and rendered electroactive via subsequent chemical functionalization.⁹¹

6.3.4 3D-printed molds for fabrication of soft bioactuators

In addition to the printing of entire bioactuators themselves, RP has been used to manufacture both sacrificial and permanent molds^{60,63,92} in which to shape bioactuators from rubber, PDMS, or other soft materials (sometimes dubbed *semi-printing*). For example, Ahn et al. created a smart material actuator, capable of bending and twisting, embedded in a soft matrix that was cast with a 3D-printed mold.⁹³ Low et al. formed silicone-based soft pneumatic grippers,⁵⁵ and Martinez et al. demonstrated a micropneumatic tentacle that could grasp and manipulate complex objects,⁵⁷ by casting soft materials into custom-printed 3D molds. An IMPC-embedded tube (cast into a 3D-printed mold) with multi-DOF capability was developed by Liu et al. to aid in minimally invasive surgical procedures.⁹⁴

Regarding actuators whose entire structures can achieve net locomotion, Jin et al. fabricated a soft robot capable of swimming, gripping, and crawling, and whose body was integrated with SMA wires and molded using 3D-printed parts.⁷⁰ Lin et al. used 3D-printed plastic molds to create a soft, rolling, coiled SMA-actuated *GoQBot*.⁹⁵ Mosadegh et al. molded a *pneu-net* (pneumatic network) soft robot whose body was actuated by air inflation.⁵⁶ Most recently, Yuk et al. published a hydraulic, polyacrylamide–alginate hydrogel actuator molded from 3D-printed solids,⁹⁶ and Wehner et al. cured an elastomer containing an embedded controller in a 3D-printed mold to fabricate a multimaterial pneumatic soft *octobot* with eight arms.⁹⁷

However, it should be noted that elastomeric bioactuators printed in whole, as compared to those cast in a 3D-printed mold, can boast easier and shorter fabrication without the subsequent need for postprocessing or assembly.⁷⁶ Morrow et al. modified a FDM printer to directly fabricate a silicone pneumatic actuator; comparison to an identical structure made from a molding process demonstrated no tradeoff in force.⁹⁸

6.4 Living bioactuators

The use of biological materials (including DNA, motor proteins, myosin–actin complexes, bacteria, algae, single cells or clusters, and natural or engineered tissues—either independently or collectively) as the primary actuators of locomotive force is still an extremely young field, but has resulted in some interesting possibilities (References^{21,99} for a review). The basic requirements of an ideal living biological actuation source include the ability to generate a controllable or repeatable force, operate under a range of environments, and be easily maintained.²⁴ Evolution has produced optimal living actuators that can operate for long term at physiological conditions (favorable for biomedical applications), wirelessly convert chemical energy (from glucose or fats, for example, which can boast energy densities up to 100 times that of a battery¹⁰) to mechanical work more efficiently than nonliving power sources, produce nontoxic and biodegradable by-products from fuel conversion, and be stimulated electro- or pharmacomechanically—thus eliminating the need for an external energy source. In addition, they are proficient at self-assembly and replication, protein synthesis, rapid adaptation (responses as short as tens of milliseconds), and are highly sensitivity to environmental conditions. Understandably, they are also biodegradable and biocompatible, and can dynamically interact with other living or nonliving components.^{15,53,99}

In general, when exploiting the innate contractility of cells or tissue to power a bioactuator, it is critical to consider the stimuli (mechanical, electrical, and biochemical) necessary for differentiation, development, or maintenance.^{46,53} RP techniques can assist in providing a suitable scaffold or environment in which appropriate cues can be tuned or added (Figure 6.2e–g). For example, a stereolithography apparatus (SLA) can print hydrogels with tissue-like stiffness values that are mechanically similar to cells' extracellular environment *in vivo*. The elastic modulus of the extracellular matrix (ECM) not only affects viability and proliferation, but also dictates the differentiation bias of cultured stem cells¹⁰⁰; for this reason, SL has been used to fabricate a variety of matrices that can realistically simulate cellular microenvironments and aid in an engineered tissue development.⁴²

Though engineered molecular^{21,24,73} and bacterial¹⁰¹ bioactuators are capable of cargo transport and fluidic pumping and can thrive in a range of environmental temperatures or pH, few have been incorporated with RP techniques.⁹⁹ The combination of many cells can allow for a collective output that is greater than the sum of its parts. Moreover, complexity (and thus functionality) increases when progressing from single cells to cell clusters (2D sheets or 3D arrangements) to tissues and systems.^{24,27} Therefore, in this section we will focus only on living eukaryotic bioactuators at the multicellular and tissue scale, comprising a synthetic mechanical scaffold and one or more actuating biological components.

Scalable molecular motors and contraction machinery that comprise muscular sarcomeres in particular can generate active contraction in multiple forms and size scales, be hierarchically combined in series or in parallel, and has evolved over millions of years with extremely high plasticity and volumetric efficiency.^{21,24,35} Functional bioactuators have been devised using whole explanted tissues,^{8,102,103} cells differentiated within a scaffold or gel,¹⁰⁴ or self-organized engineered tissue.¹⁰⁵ It is worth noting that although smooth muscle¹⁰⁶ is capable of force production, its relatively slow contraction has prohibited its employment in biorobots that require rapid actuation.⁹⁹

6.4.1 Cardiac muscle

Cardiomyocytes (cardiac muscle cells) provide an excellent source for bioactuation due to their intrinsic, synchronous contraction; thus, external stimulation is unnecessary. The cells can form a syncytium through gap junctions and cell–cell adhesions, and produce spontaneous contractions.^{21,27} The original developments in cardiac-based bioactuators included locomotive machines such as walking microrobots,^{5,107,108} on-chip pumps,¹⁰⁹ and swimming robots¹¹⁰ and *jellyfish*.⁹ However, most were constructed on silicon or PDMS substrates that did not mimic the native cellular microenvironment nor allow for dynamic adaptation; few have been coupled to substrates fabricated from RP methods.

To demonstrate the ability of cardiac cells to induce the locomotion of a material with an elastic modulus similar to that of the native myocardium, Chan et al. developed a biological robot (dubbed *biobot*) from polyethylene glycol (PEG) hydrogel.^{41,111} A modified SLA was utilized to fabricate a microcantilever, the surface of which was functionalized with collagen to adhere a culture of primary neonatal rat cardiomyocytes. To transform this hybrid structure into a bioactuator capable of directional locomotion, a net asymmetry of actuation was introduced in the cantilever design. Furthermore, the thickness of the cantilever was optimized to control the curvature of the actuating leg and maximize the locomotive speed. The occurrence of a *power stroke* induced by rhythmic spontaneous cardiac sheet contraction drove the actuating leg to bend downward, increasing the friction and causing the biobot to propel forward. With a maximum velocity of 236 $\mu\text{m/s}$, the cardiac biohybrid actuator demonstrated efficient mechanisms of autonomous locomotion and a novel approach to spatially control biochemical and physical cues during fabrication.

6.4.2 Skeletal muscle

The behavioral complexity and degree of external control that can be imposed on cardiac muscle are limited by its intrinsic spontaneous contractility. The ability to regulate an actuator through the modulation of an applied stimulus not only

allows for precise control over its motion but also opens up avenues for greater functionality. Skeletal muscle is the primary generator of animal locomotion, with a dense structure comprising a modular hierarchy with an arrangement of motor units that can be recruited individually or in summation. It exhibits a greater force-to-weight ratio in comparison to many rigid mechanical actuators.^{21,24,27,99} A high degree of spatial and temporary control over actuation, even of single fibers, is possible via external sources such as electrical,² optical,^{112,113} or neural^{114,115} stimulation.

A skeletal muscle-powered biobot developed by Cvetkovic et al. mimicked the mammalian musculoskeletal system, wherein muscle contraction drives the articulation of bones across flexible joints.^{2,116} A 3D-printed skeleton (comprised of a flexible beam connected to two stiff pillars) was fabricated using a SLA and subsequently anchored to an engineered muscle strip containing differentiating C2C12 myoblasts. The 3D muscle strip contained natural ECM hydrogels (fibrin and Matrigel™) that supported the densely embedded cells. To induce the locomotion-driving contraction of the muscle strip, the biobot was positioned within an electric field and subjected to a pulse stimulation of 1–4 Hz, resulting in a global response of the excitable cells. The introduction of deliberate asymmetry in the pillars (achievable with a slight modification in the rapid prototyping technique) allowed the biobot to move in a unidirectional trajectory along a surface in a fluid with a maximum locomotion of ~150 μm/s.

Although the muscle strip successfully induced net locomotion, the muscle was permanently tethered to the skeleton, preventing the adaptation of the muscle to other skeleton structures.² A second iteration by Raman et al. was devised with a muscle ring structure, formed in a separate 3D-printed mold before being transferred to the skeleton.¹¹³ Muscle rings exhibited higher myofiber alignment and increased viability.¹¹⁶ The C2C12s were also genetically modified to express Channel rhodopsin (ChR2), a membrane protein that causes muscle contraction under the presence of blue light.¹¹⁷ This allowed for the control of locomotion through an optical stimulus, which could be positioned on localized regions of the biobot, thus enabling the development of a symmetrical yet bidirectional bio-actuator whose direction of locomotion was determined by which ring the light stimulated. Similarly, a single device could also be forced to rotate by stimulating only one half of a single muscle ring. To maximize force production, the biobots underwent an *exercise regimen* of daily optical stimulation throughout muscle differentiation. Exercise was shown to improve myotube formation, leading to an increased tension and locomotive speed up to 310 μm/s.

6.4.3 Control mechanisms for living bioactuators

Cell- and tissue-based bioactuators can be controlled in a variety of manners, both internally regulated and externally applied. Internal, cell-based control

utilizes intrinsic sensory pathways or mechanisms within the biological material. External, noncell-based control involves remote operation or local environmental stimuli—whether chemical, magnetic, electrical, optical, or a combination thereof.²⁴ Cardiomyocytes, for example, can be controlled with temperature variance, and skeletal muscle cells can be activated via electrical fields, optogenetics, or a neuronal network (requiring acetylcholine release from an innervating motor neuron). Optical control is negligibly invasive, irreversible, and can provide precise spatiotemporal control. It can also be used as an on/off toggle switch to quickly modulate contraction, pacing, or net actuation.^{21,118,119}

The ability to noninvasively control living bioactuators with such specificity sets the stage for the development of future biological machines for a variety of applications. However, care must be taken to ensure that stimuli are applied within ranges that are acceptable or minimally invasive for living biological material, especially when dealing with exposure to ultraviolet (UV) light or electromagnetic fields, changes in pH and temperature, media electrolysis, and toxic waste by-products.²⁴

6.5 Applications

Rapid prototyping technologies, which continue to advance in efficiency, resolution, and biocompatible material selection,^{72,120} provide a controlled, economical, and potentially high-throughput solution to the production of responsive bioactuators for myriad applications. The extreme diversity of fabrication approaches, material composition, and functionalities suggest that soft bioactuators can be used in a variety of manners and systems. They boast many useful capabilities, including shape deformation, conformation, and sensitivity to their surroundings, movement in unstructured environments, and manipulation of delicate objects.⁵⁷ Their scalability also enables operation in environments where movement of their larger counterparts would be impractical or impossible. Furthermore, these devices are lighter, undergo more continuous and natural deformation with simple control inputs, and are more easily mass produced than their motor-driven counterparts.¹²¹

It is expected that custom-printed 3D robots and actuator structures will appear in application areas as diverse as devices for human–computer interaction, chemical and environmental remediation, or surgical tools for training.⁸⁸ For example, Alblalaid et al. used a projection microstereolithography system to 3D-print polymer components on top of which thin metals could be coated to create a microscale gripper. The gripper was thermoelectrically activated and could be used for surgical manipulation.¹²² Also, applications are being explored with fluid-powered actuators demonstrating human potential in macroscale self-healing medical implants or wearable orthotics.^{18,123} Park et al. designed an artificial

soft biorobotic pneumatic muscle actuator attached to a 3D-printed leg model that could be worn over the knee,¹²⁴ and Doncieux et al. developed a bioinspired *Gummi Arm* fabricated of 3D-printed plastic structures connected by agonist–antagonist joints that mimicked soft tendons.¹²⁵

Bioactuators with or without cells might be designed for drug screening or delivery,¹²⁶ bioreactors or lab-on-a-chip devices, vascular pumps and monitors, or adaptive prosthetics.^{24,27,53} Some aspects of this technology have been proposed for use as part of drug delivery systems where a drug could detect body-site specific temperatures during local infection or low-pH tumor environments, and intelligently self-locate and self-release pharmaceuticals.⁷¹ Independently or in large numbers, these actuators might also be programmed to form mobile and robust sensor and communication networks, allowing them to work in rubble fields, utility conduits, or the ocean floor to assist work in multiple industries.^{127,128} Terrestrial actuators that could navigate dense environments and change shape, color, or surface temperature for camouflage would fit in well with outdoor research or military operations.⁷⁰

Both living and nonliving bioactuators must be designed such that the form matches the intended function.^{46,99,129} When building with muscle, for example, the devices might need to closely match the performance of their *in vivo* equivalents, especially if their intended use is to mimic a native tissue for a drug testing or regenerative application, or to provide a novel platform for understanding fundamental biological phenomena. However, when physiological relevance is less important to the end objective, replication of natural performance might be overlooked in favor of maximal efficiency, contractility, or power output. For example, primary cardiomyocytes might be overlooked in favor of a skeletal muscle cell line if extensive scaling-up or wider contractile ranges were necessary to the functionality of the bioactuator, but favored in certain temperatures or situations requiring cellular synchrony.

6.6 Limitations and future directions

Though much progress has been made, significant fundamental challenges still remain. Soft bioactuators must be manufactured in a manner that preserves the mechanical integrity of their structure and allows shock absorption, deformation, flexibility, and minimal damage. A major challenge is attempting to increase force output from elastic or synthetic systems without compromising the biomimetic properties that promote integration with living materials or the complexity of design and function, which characterizes the natural world.^{15,18,19,40,120} When integrating living biomaterials, researchers must consider scenarios, which might enhance or hinder force production—for example, cellular viability, (self-)adhesion, organization, alignment, directionality, and overall structure, which all contribute

significantly to function. Moreover, cells must be maintained in cell culture media at highly regulated conditions ensuring nutrient and oxygen delivery, with the application of appropriate external cues for guidance of tissue development and mechanical performance. They are subject to a variety of failure modes, including mechanical (within the tissue or at the interface), metabolic, fatigue, damage or injury, and necrosis.^{24,53} Finally, it can also be difficult to model the active and passive response of a cellular or living system in uncertain environmental conditions. Researchers still lack a deep understanding of how fundamental processes of cells (such as local interactions) function globally across length scales.²⁷

Thus far, 3D-printed actuators have been assembled mostly with singular modalities. However, just as traditional robots contain multiple systems for various modalities (actuation, perception, computation, power, etc.), future designs of soft bioactuators should integrate multiple components and functionalities—including sensing^{121,130} and processing of information—as well as multiple cell types or materials to achieve more complex, precise, and useful actuation.^{52,99} In the future, it will be necessary to implement feedback systems and control mechanisms that help to extend the lifetime, precision, repeatability, and outputs of bioactuators, while also enhancing their operation outside encapsulated or restricted environmental conditions.^{24,35,131,132} With living bioactuators, coculture systems can provide a synergistic support system to enhance the overall performance. To meet metabolic demands of living cells on larger size (>0.5 mm) or time scales, a vascular component will be necessary for consistent nutrient delivery. Indeed, the lack of perfusive blood vessels within regenerated tissues has historically plagued developments in the TE field. Guo, Miller, and Kolesky all have demonstrated various 3D-bioprinting methods that could be used in microvascular network formation.^{133–135} In addition, innervation of muscle fibers with motor neurons can aid in the preservation of skeletal muscle phenotype, while allowing for better control, directed motion, or more complex functional outputs.^{46,53}

With regards to fabrication, some limitations exist within the realm of technological advancements that might permit the construction of specific structures applicable to bioactuators. In the future, researchers will need to consider how to develop materials and scaffolds that can support cell and tissue outputs with maximal efficiency or power. This may include specific nano- or microscale geometries, shorter print times for large structures, higher resolution, greater range of material properties, improvement of surface adhesion techniques, and attachment of ECM.^{29,35} Moreover, there exists a demand for multimaterial rapid prototyping advancements that could combine properties of multiple materials in one product.^{11,36,51} Soft bioactuators might integrate new techniques in 3D printing of layered fabrics,^{136,137} SMPs for flexible electronics,⁸⁰ and direct printing of electronic fluidic components¹³⁸ for onboard automation. From any perspective, these advancements represent a rapidly growing field with the potential to significantly benefit human life.

References

1. Umedachi T, Vikas V, Trimmer BA. Softworms: The design and control of non-pneumatic, 3D-printed, deformable robots. *Bioinspir Biomim* 2016; **11**. doi:10.1088/1748-3190/11/2/025001.
2. Cvetkovic C, Raman R, Chan V, Williams BJ, Tolish M, Bajaj P et al. Three-dimensionally printed biological machines powered by skeletal muscle. *Proc Natl Acad Sci USA* 2014; **111**: 10125–10130.
3. Kováč M. The bioinspiration design paradigm: A perspective for soft robotics. *Soft Robot* 2013; **1**: 28–37.
4. Bartlett NW, Tolley MT, Overvelde JTB, Weaver JC, Mosadegh B, Bertoldi K et al. A 3D-printed, functionally graded soft robot powered by combustion. *Science* 2015; **349**: 161–165.
5. Feinberg AW, Feigel A, Shevkoplyas SS, Sheehy S, Whitesides GM, Parker KK. Muscular thin films for building actuators and powering devices. *Science* 2007; **317**: 1366–1370.
6. Wen L, Wang T, Wu G, Liang J. Quantitative thrust efficiency of a self-propulsive robotic fish: Experimental method and hydrodynamic investigation. *IEEE/ASME Trans Mechatronics* 2013; **18**: 1027–1038.
7. Low KH. Design, development and locomotion control of bio-fish robot with undulating anal fins. *Int J Robot Autom* 2007; **22**: 88–99.
8. Herr H, Dennis RG. A swimming robot actuated by living muscle tissue. *J Neuroeng Rehabil* 2004; **1**: 1–9.
9. Nawroth JC, Lee H, Feinberg AW, Ripplinger CM, McCain ML, Grosberg A et al. A tissue-engineered jellyfish with biomimetic propulsion. *Nat Biotechnol* 2012; **30**: 792–797.
10. Majidi C. Soft robotics: A perspective—current trends and prospects for the future. *Soft Robot* 2013; **1**: 5–11.
11. Gui JZ, Yang B-S, Yang YJ, Chang DEE, Choi KH, Gul JZ et al. In situ UV curable 3D printing of multi-material tri-legged soft bot with spider mimicked multi- step forward dynamic gait. *Smart Mater Struct* 2016; **25**: 1–12.
12. Vincent JFV, Bogatyrev OA, Bogatyrev NR, Bowyer A, Pahl A-K. Biomimetics: Its practice and theory. *J R Soc Interface* 2006; **3**: 471–482.
13. Full RJ. Using biological inspiration to build artificial life that locomotes. *EvoWorkshops* 2001; **2217**: 110–120.
14. Ricotti L, Menciassi A. Bio-hybrid muscle cell-based actuators. *Biomed Microdevices* 2012; **14**: 987–998.
15. Kim S, Laschi C, Trimmer B. Soft robotics: A bioinspired evolution in robotics. *Trends Biotechnol* 2013; **31**(5): 1–8.
16. Tadesse Y, Wu L, Saharan LK. Musculoskeletal system for bio-inspired robotic systems. *Focus Dyn Syst Control* 2016; **138**: 11–17.
17. Bernabei R, Power J. On three categories of conscious machines. In: Lepora NF, Mura A, Mangan M, Verschure PFMJ, Desmulliez M, Prescott TJ (Eds.). *Biomimetic and Biohybrid Systems: Proceedings of the 5th International Conference, Living Machines 2016, Edinburgh, UK*. Springer International Publishing, 2016, pp. 40–47.
18. Zolfagharian A, Kouzani AZ, Khoo SY, Gibson I, Kaynak A. 3D printed hydrogel soft actuators. In: *IEEE Region 10 Conference (TENCON)*. 2016, pp. 2274–2279.
19. Lee C, Kim M, Kim YJ, Hong N, Ryu S, Kim HJ et al. Soft robot review. *Int J Control Autom Syst* 2017; **15**: 3–15.
20. Inoue D, Kabir AR, Sada K, Gong JP. Tissue engineering approach to making soft actuators. *Soft Actuators* 2014; 475–487. doi:10.1007/978-4-431-54767-9_33.

21. Chan V, Asada HH, Bashir R. Utilization and control of bioactuators across multiple length scales. *Lab Chip* 2014; **14**: 653–670.
22. Burgert I, Fratzl P. Actuation systems in plants as prototypes for bioinspired devices. *Philos Trans A Math Phys Eng Sci* 2009; **367**: 1541–1557.
23. Kabumoto K, Hoshino T, Akiyama Y, Morishima K. Voluntary movement controlled by the surface EMG signal for tissue-engineered skeletal muscle on a gripping tool. *Tissue Eng Part A* 2013; **19**: 1695–1703.
24. Carlsen RW, Sitti M. Bio-hybrid cell-based actuators for microsystems. *Small* 2014; **10**: 3831–3851.
25. Bowers AE, Rossiter JM, Walters PJ, Ieropoulos IA. Dielectric elastomer pump for artificial organisms. *Proc SPIE* 2011; **7976**: 797629.
26. Cho K-J, Wood R. Biomimetic robots. In: *Springer Handbook of Robotics*. 2016, pp. 543–574.
27. Kamm RD, Bashir R. Creating living cellular machines. *Ann Biomed Eng* 2013; **42**: 445–459.
28. Prinz F, Atwood C, Aubin R, Beaman J, Brown R, Fussell P et al. JTEC/WTEC Panel Report on Rapid prototyping in Europe and Japan. 1997.
29. Hinton TJ, Lee A, Feinberg AW. 3D bioprinting from the micrometer to millimeter length scales: Size does matter. *Curr Opin Biomed Eng* 2017. doi:10.1016/j.cobme.2017.02.004.
30. Lantada AD, Morgado PL. Rapid prototyping for biomedical engineering: Current capabilities and challenges. *Annu Rev Biomed Eng* 2012; **14**: 73–96.
31. Peltola S, Melchels FPW, Grijpma DW, Kellomäki M. A review of rapid prototyping techniques for tissue engineering purposes. *Ann Med* 2008; **40**: 268–280.
32. Stanek M, Manas D, Manas M, Navratil J, Kyas K, Senkerik V et al. Comparison of different rapid prototyping methods. *Int J Math Comput Simul* 2012; **6**: 550–557.
33. Upcraft S, Fletcher R. The rapid prototyping technologies. *Assem Autom* 2003; **23**: 318–330.
34. Bhatt R, Tang CP, Lee L-F, Krovi V. Web-based self-paced virtual prototyping tutorials. *Proc DETC* 2003; 1–7.
35. Lantada AD. Cell-based sensors and cell-based actuators. In: Díaz Lantada A (Ed.). *Microsystems for Enhanced Control of Cell Behavior*. Springer International Publishing: Cham, Switzerland, 2016, pp. 373–386.
36. Keating SJ, Gariboldi MI, Patrick WG, Sharma S, Kong DS, Oxman N. 3D printed multimaterial microfluidic valve. *PLoS One* 2016; **11**: 1–13.
37. Munaz A, Vadivelu RK, John JS, Barton M, Kamble H, Nguyen N. Three-dimensional printing of biological matters. *J Sci Adv Mater Devices* 2016; **1**: 1–17.
38. Khademhosseini A, Langer R. A decade of progress in tissue engineering. *Nat Protoc* 2016; **11**: 1775–1781.
39. Yeong W-Y, Chua C-K, Leong K-F, et al. Rapid prototyping in tissue engineering: Challenges and potential. *Trends Biotechnol* 2004; **22**: 643–652.
40. Zhu W, Castro NJ, Zhang LG. Nanotechnology and 3D bioprinting for neural tissue regeneration. In: *3D Bioprinting and Nanotechnology in Tissue Engineering and Regenerative Medicine*. Elsevier, London, UK, 2015, pp. 307–331.
41. Chan V, Jeong JH, Bajaj P, Collens MB, Saif T, Kong H et al. Multi-material bio-fabrication of hydrogel cantilevers and actuators with stereolithography. *Lab Chip* 2012; **12**: 88–98.
42. Arcaute K, Mann BK, Wicker RB. Practical use of hydrogels in stereolithography for tissue engineering applications. In: Bártolo PJ (Ed.). *Stereolithography: Materials, Processes and Applications*. Springer, Boston, MA, 2011, pp. 299–331.
43. Zorlutuna P, Jeong JH, Kong H, Bashir R. Stereolithography-based hydrogel microenvironments to examine cellular interactions. *Adv Funct Mater* 2011; **21**: 3642–3651.

44. Bajaj P, Chan V, Jeong JH, Zorlutuna P, Kong H, Bashir R. 3-D biofabrication using stereolithography for biology and medicine. In: *Engineering in Medicine and Biology Society (EMBC), 2012 Annual International Conference of the IEEE*. 2012, pp. 6805–6808.
45. Melchels FPW, Feijen J, Grijpma DW. A review on stereolithography and its applications in biomedical engineering. *Biomaterials* 2010; **31**: 6121–6130.
46. Uzel SGM, Pavesi A, Kamm RD. Microfabrication and microfluidics for muscle tissue models. *Prog Biophys Mol Biol* 2014; **115**: 279–293.
47. Bajaj P, Schweller RM, Khademhosseini A, West JL, Bashir R. 3D biofabrication strategies for tissue engineering and regenerative medicine. *Annu Rev Biomed Eng* 2014; **16**: 247–276.
48. Burg T, Cass CAP, Groff R, Pepper M, Burg KJL. Building off-the-shelf tissue-engineered composites. *Philos Trans A Math Phys Eng Sci* 2010; **368**: 1839–1862.
49. Ahn SH, Lee J, Park SA, Kim WD. Three-dimensional bio-printing equipment technologies for tissue engineering and regenerative medicine. *Tissue Eng Regen Med* 2016; **13**: 663–676.
50. Pfister A, Landers R, Laib A, Hübner U, Schmelzeisen R, Mülhaupt R. Biofunctional rapid prototyping for tissue-engineering applications: 3D bioplotting versus 3D printing. *J Polym Sci Part A Polym Chem* 2004; **42**: 624–638.
51. Zolfagharian A, Kouzani AZ, Khoo SY, Moghadam AAA, Gibson I, Kaynak A. Evolution of 3D printed soft actuators. *Sensors Actuators, A Phys* 2016; **250**: 258–272.
52. Rus D, Tolley MT. Design, fabrication and control of soft robots. *Nature* 2015; **521**: 467–475.
53. Dennis RG, Herr H. Engineered muscle actuators: Cells and tissues. In: Bar-Cohen Y (Ed.). *Biomimetics: Biologically Inspired Technologies*. CRC Press, Boca Raton, FL, 2005, pp. 243–266.
54. Schulte HF. The characteristics of the McKibben artificial muscle. In: *The Application of External Power in Prosthetics and Orthotics*. National Acadamey of Sciences-National Research Council, Washington DC, 1961, pp. 94–102.
55. Low J-H, Yeow C-H. Rod-based fabrication of customizable soft robotic pneumatic gripper devices for delicate tissue manipulation. *J Vis Exp* 2016; **114**: e54175.
56. Mosadegh B, Polygerinos P, Keplinger C, Wennstedt S, Shepherd RF, Gupta U et al. Pneumatic networks for soft robotics that actuate rapidly. *Adv Funct Mater* 2014; **24**: 2163–2170.
57. Martinez RV, Branch JL, Fish CR, Jin L, Shepherd RF, Nunes RMD et al. Robotic tentacles with three-dimensional mobility based on flexible elastomers. *Adv Mater* 2013; **25**: 205–212.
58. Daerden F, Lefeber D. Pneumatic artificial muscles: Actuators for robotics and automation. *Eur J Mech Environ Eng* 2002; **47**: 11–21.
59. Shepherd RF, Ilievski F, Choi W, Morin SA, Stokes AA, Mazzeo AD et al. Multigait soft robot. *Proc Natl Acad Sci USA* 2011; **108**: 20400–20403.
60. Ilievski F, Mazzeo AD, Shepherd RF, Chen X, Whitesides GM. Soft robotics for chemists. *Angew Chemie - Int Ed* 2011; **50**: 1890–1895.
61. Suzumori K, Iikura S, Tanaka H. Applying a flexible microactuator to robotic mechanisms. *IEEE Control Syst* 1992; **12**: 21–27.
62. Caldwell DG, Tsagarakis N, Medrano-Cerda GA. Bio-mimetic actuators: Polymeric pseudo muscular actuators and pneumatic uscle actuators for biological emulation. *Mechatronics* 2000; **10**: 499–530.
63. Robertson MA, Sadeghi H, Florez JM, Paik J. Soft pneumatic actuator fascicles for high force and reliability. *Soft Robot* 2016; **4**: 23–32.

64. Yang Y, Chen Y. Novel design and 3D printing of variable stiffness robotic fingers based on shape memory polymer. In: *6th IEEE RAS/EMBS International Conference on Biomedical Robotics and Biomechatronics (BioRob) June 26–29, 2016*. Singapore, 2016, pp. 195–200.
65. Wang Z, Hirai S. A 3D printed soft gripper integrated with curvature sensor for studying soft grasping. In: *Proceedings of the 2016 IEEE/SICE International Symposium on System Integration*. IEEE: Sapporo, Japan, 2016, pp. 629–633.
66. Polygerinos P, Wang Z, Galloway KC, Wood RJ, Walsh CJ. Soft robotic glove for combined assistance and at-home rehabilitation. *Rob Auton Syst* 2015; **73**: 135–143.
67. MacCurdy R, Katzschmann R, Kim Y, Rus D. Printable hydraulics: A method for fabricating robots by 3D co-printing solids and liquids. In: *2016 IEEE International Conference on Robotics and Automation (ICRA)*. 2016.
68. Underwater propulsion from a 3D printer. *Phys Org* 2013. Available: <https://phys.org/news/2013-07-underwater-propulsion-3d-printer.html>.
69. Katzschmann RK, Marchese AD, Rus D. Hydraulic autonomous soft robotic fish for 3D swimming. In: Kumar V, Khatib O, Hsieh MA (Eds.). *Experimental Robotics*. Springer Tracts in Advanced Robotics, 2000, pp. 149–163.
70. Jin H, Dong E, Xu M, Liu C, Alici G, Jie Y. Soft and smart modular structures actuated by shape memory alloy (SMA) wires as tentacles of soft robots. *Smart Mater Struct* 2016; **25**: 85026.
71. Mano JF. Stimuli-responsive polymeric systems for biomedical applications. *Adv Eng Mater* 2008; **10**: 515–527.
72. Sydney Gladman A, Matsumoto EA, Nuzzo RG, Mahadevan L, Lewis JA. Biomimetic 4D printing. *Nat Mater* 2016; **15**: 413–418.
73. Knoblauch M, Peters WS. Biomimetic actuators: Where technology and cell biology merge. *Cell Mol Life Sci* 2004; **61**: 2497–2509.
74. Bakarich SE, Gorkin R, Panhuis M In Het, Spinks GM. 4D printing with mechanically robust, thermally actuating hydrogels. *Macromol Rapid Commun* 2015; **36**: 1211–1217.
75. Ge Q, Qi HJ, Dunn ML. Active materials by four-dimension printing. *Appl Phys Lett* 2013; **103**. doi:10.1063/1.4819837.
76. Walters P, McGoran D. Digital fabrication of ‘smart’ structures and mechanisms-creative applications in art and design. In: *International Conference on Digital Printing Technologies and Digital Fabrication 2011*. Minneapolis, 2011, pp. 185–188.
77. Umedachi T, Trimmer BA. Design of a 3D-printed soft robot with posture and steering control. In: *2014 IEEE International Conference on Robotics & Automation (ICRA)*. Hong Kong, 2014, pp. 2874–2879.
78. Umedachi T, Vikas V, Trimmer BA. Highly deformable 3-D printed soft robot generating inching and crawling locomotions with variable friction legs. In: *2013 IEEE/RSJ International Conference on Intelligent Robots and Systems (IROS)*. Tokyo, 2013, pp. 4590–4595.
79. Mao Y, Yu K, Isakov MS, Wu J, Dunn ML, Jerry Qi H. Sequential self-folding structures by 3D printed sigital shape memory polymers. *Sci Rep* 2015; **5**: 13616.
80. Zarek M, Layani M, Cooperstein I, Sachyani E, Cohn D, Magdassi S. 3D printing of shape memory polymers for flexible electronic devices. *Adv Mater* 2016; **28**: 4449–4454.
81. Bodaghi M, Damanpack AR, Liao WH. Self-expanding/shrinking structures by 4D printing. *Smart Mater Struct* 2016; **25**: 105034.
82. Mao Y, Ding Z, Yuan C, Ai S, Isakov M, Wu J et al. 3D printed reversible shape changing components with stimuli responsive materials. *Sci Rep* 2016; **6**: 24761.
83. Wu J, Yuan C, Ding Z, Isakov M, Mao Y, Wang T et al. Multi-shape active composites by 3D printing of digital shape memory polymers. *Sci Rep* 2016; **6**. doi:10.1038/srep24224.

84. Zolfagharian A, Kouzani AZ, Nasri-Nasrabadi B, Adams S, Yang Khoo S, Norton M et al. 3D printing of a photo-thermal self-folding actuator. In: *DesTech Conference Proceedings: The International Conference on Design and Technology*. KEG, 2017, pp. 15–22.
85. Zhu W, Li J, Leong YJ, Rozen I, Qu X, Dong R et al. 3D-printed artificial microfish. *Adv Mater* 2015; **27**: 4411–4417.
86. Bar-Cohen Y. Biologically inspired intelligent robots using artificial muscles. *Strain* 2005; **41**: 19–24.
87. Asaka K, Nakamura K. Current status of applications and markets of soft actuators. In: Asaka K, Okuzaki H (Eds.). *Soft Actuators*. Springer Japan, 2014, pp. 19–30.
88. Rossiter J, Walters P, Stoimenov B. Printing 3D dielectric elastomer actuators for soft robotics. In: Bar-Cohen Y, Wallmersperger T (Eds.). *Electroactive Polymer Actuators and Devices (EAPAD) 2009*. SPIE, 2009. doi:10.1117/12.815746.
89. Cai J, Vanhorn A, Mullikin C, Stabach J, Alderman Z. 4D Printing of soft robotic facial muscles. In: *International Solid Freeform Fabrication (SFF) Symposium – An Additive Manufacturing Conference*. Austin, 2015, pp. 1537–1553.
90. Nguyen CT, Phung H, Jung H, Kim U, Nguyen TD, Park J et al. Printable monolithic hexapod robot driven by soft actuator. *2015 IEEE Int Conf Robot Autom* 2015; 4484–4489.
91. Carrico JD, Traeden NW, Aureli M, Leang KK. Fused filament 3D printing of ionic polymer-metal composites (IPMCs). *Smart Mater Struct* 2015; **24**: 125021.
92. Wei T, Stokes A, Webb B. A soft pneumatic maggot robot. *Living Mach* 2016; **1**: 375–386.
93. Ahn S, Lee K, Kim H, Wu R, Kim J, Song S. Smart soft composite: An integrated 3D soft morphing structure using bend-twist coupling of anisotropic materials. *Int J Precis Eng Manuf* 2012; **13**: 631–634.
94. Liu J, Wang Y, Zhao D, Zhang C, Chen H, Li D. Design and fabrication of an IPMC-embedded tube for minimally invasive surgery applications. In: Bar-Cohen Y (Ed.). *Electroactive Polymer Actuators and Devices (EAPAD)*. 2014, p. 90563K.
95. Lin H-T, Leisk GG, Trimmer B. GoQBot: A caterpillar-inspired soft-bodied rolling robot. *Bioinspir Biomim* 2011; **6**. doi:10.1088/1748-3182/6/2/026007.
96. Yuk H, Lin S, Ma C, Takaffoli M, Fang NX, Zhao X et al. Hydraulic hydrogel actuators and robots optically and sonically camouflaged in water. *Nat Commun* 2017; **8**: 14230.
97. Wehner M, Truby RL, Fitzgerald DJ, Mosadegh B, Whitesides GM, Lewis JA et al. An integrated design and fabrication strategy for entirely soft, autonomous robots. *Nature* 2016; **536**: 451–455.
98. Morrow J, Hemleben S, Menguc Y. Directly fabricating soft robotic actuators with an open-source 3-D printer. *IEEE Robot Autom Lett* 2016; **2**: 277–281.
99. Feinberg AW. Biological soft robotics. *Annu Rev Biomed Eng* 2015; **17**: 243–265.
100. Engler AJ, Griffin MA, Sen S, Bönnemann CG, Sweeney HL, Discher DE. Myotubes differentiate optimally on substrates with tissue-like stiffness: Pathological implications for soft or stiff microenvironments. *J Cell Biol* 2004; **166**: 877–887.
101. Stanton MM, Park B-W, Miguel-López A, Ma X, Sitti M, Sánchez S. Biohybrid micro-tube swimmers driven by single captured bacteria. *Small* 2017; **13**: 1603679.
102. Akiyama Y, Hoshino T, Iwabuchi K, Morishima K. Room temperature operable autonomously moving bio-microrobot powered by insect dorsal vessel tissue. *PLoS One* 2012; **7**: e38274.
103. Webster VA et al. *Aplysia californica* as a novel source of material for biohybrid robots and organic machines. In: Lepora N, Mura A, Mangan M, Verschure P, Desmulliez M, Prescott T (Eds.). *Biomimetic and Biohybrid Systems. Living Machines 2016: Lecture Notes in Computer Science*, vol. 9793. Springer, Cham, 2016, pp. 268–279.

104. Hinds S, Bian W, Dennis RG, Bursac N. The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials* 2011; **32**: 3575–3583.
105. Dennis RG, Kosnik PE. Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro. *Vitr Cell Dev Biol - Anim* 2000; **36**: 327–335.
106. Duan B, Hockaday LA, Kang KH, Butcher JT. 3D Bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *J Biomed Mater Res Part A* 2012; **101**(5): 1–10.
107. Park J, Ryu J, Choi SK, Seo E, Cha JM, Ryu S et al. Real-time measurement of the contractile forces of self-organized cardiomyocytes on hybrid biopolymer microcantilevers. *Anal Chem* 2005; **77**: 6571–6580.
108. Kim J, Park J, Yang S, Baek J, Kim B, Lee SH et al. Establishment of a fabrication method for a long-term actuated hybrid cell robot. *Lab Chip* 2007; **7**: 1504–1508.
109. Tanaka Y, Morishima K, Shimizu T, Kikuchi A, Yamato M, Okano T et al. An actuated pump on-chip powered by cultured cardiomyocytes. *Lab Chip* 2006; **6**: 362–368.
110. Williams BJ, Anand SV, Rajagopalan J, Saif MTA. A self-propelled biohybrid swimmer at low Reynolds number. *Nat Commun* 2014; **5**: 3081.
111. Chan V, Park K, Collens MB, Kong H, Saif TA, Bashir R. Development of miniaturized walking biological machines. *Sci Rep* 2012; **2**: 1–8.
112. Park S, Gazzola M, Park KS, Park S, Dauth S, Capulli AK et al. Phototactic guidance of a tissue-engineered soft-robotic ray. *Science (80-)* 2016; **353**: 158–162.
113. Raman R, Cvetkovic C, Uzel SGM, Platt RJ, Sengupta P, Kamm RD. Optogenetic skeletal muscle-powered adaptive biological machines. *Proc Nat Acad Sci USA* 2016. doi:10.1073/pnas.1516139113.
114. Uzel SGM, Platt RJ, Subramanian V, Pearl TM, Rowlands CJ, Chan V et al. Microfluidic device for the formation of optically excitable, three-dimensional, compartmentalized motor units. *Sci Adv* 2016; **2**: e1501429.
115. Cvetkovic C, Rich MH, Raman R, Kong H, Bashir R. A 3D-printed platform for modular neuromuscular motor units. *Microsystems Nanoeng* 2017; **3**. doi:10.1038/micronano.2017.15.
116. Raman R, Cvetkovic C, Bashir R. A modular approach to the design, fabrication, and characterization of muscle-powered biological machines. *Nat Protoc* 2017; **12**: 519–533.
117. Sakar MS, Neal D, Boudou T, Borochin MA, Li Y, Weiss R et al. Formation and optogenetic control of engineered 3D skeletal muscle bioactuators. *Lab Chip* 2012; **12**: 4976–4985.
118. Uzel SGM, Platt RJ, Subramanian V, Pearl TM, Rowlands CJ, Chan V et al. Microfluidic device for the formation of optically motor units. *Sci Adv* 2016; **2**: e1501429.
119. Chan V, Neal DM, Uzel SGM, Kim H, Bashir R, Asada HH. Fabrication and characterization of optogenetic, multi-strip cardiac muscles. *Lab Chip* 2015; **15**: 2258–2268. doi:10.1039/C5LC00222B.
120. Kapsali V, Toomey A, Oliver R, Tandler L. Biomimetic spatial and temporal (4D) design and fabrication. In: Lepora NF, Mura A, Krapp HG, Verschure PFMJ, Prescott TJ (Eds.). *Biomimetic and Biohybrid Systems: Second International Conference, Living Machines 2013, London, UK, July 29–August 2, 2013, Proceedings*. Springer, Berlin, Germany, 2013, pp. 387–389.
121. Zhao H, O'Brien K, Li S, Shepherd RF, O'brien K, Li S et al. Optoelectronically innervated soft prosthetic hand via stretchable optical waveguides. *Sci Robot* 2016; **7529**: eaai7529.

122. Alblalaid K, Overton J, Lawes S, Kinnell P. A 3D-printed polymer micro-gripper with self-defined electrical tracks and thermal actuator. *J Micromechanics Microengineering* 2017; **27**: 45019.
123. Wirekoh H, Park Y-L. Design of flat pneumatic artificial muscles. *Smart Mater Struct* 2017; **26**: 1–10.
124. Park YL, Santos J, Galloway KG, Goldfield EC, Wood RJ. A soft wearable robotic device for active knee motions using flat pneumatic artificial muscles. In: *2014 IEEE International Conference on Robotics & Automation (ICRA)*. IEEE, Hong Kong, 2014, pp. 4805–4810.
125. Doncieux S, Girard B, Guillot A, Hallam J, Meyer J-A, Mouret J-B. Co-exploring actuator antagonism and bio-inspired control in a printable robot arm. *11th Int Conf Simul Adapt Behav SAB 2010, Paris - Clos Lucé, Fr August 25–28, 2010 Proc* 2010; **1**: 662.
126. Vandenburgh H. High-content drug screening with engineered musculoskeletal tissues. *Tissue Eng Part B Rev* 2010; **16**: 55–64.
127. Jung Y, Bae J. A six-legged walking robot bio-inspired walking pattern: Kinematic analysis. In: Lee J, Lee MC, Liu H, Ryu J (Eds.). *ICIRA 2013: Intelligent Robotics and Applications. Lecture Notes in Computer Science*. Springer, Berlin, Germany, 2013, pp. 257–264.
128. Schuldt DW, Rife J, Trimmer BA, Saunders F, Trimmer BA, Rife J et al. Softworms: The design and control of non-pneumatic, 3D-printed, deformable robots. *Bioinspir Biomim* 2016; **11**: 1–16.
129. Kim J, Kim HN, Lang Y, Pandit A. Biologically inspired micro- and nanoengineering systems for functional and complex tissues. *Tissue Eng Part A* 2014; **19**: 1–4.
130. Katz E. Biomolecular logic systems: Applications to biosensors and bioactuators. In: Cullum BM, McLamore ES (Eds.). *Smart Biomedical and Physiological Sensor Technology XI*. SPIE, 2014. doi:10.1117/12.2049959.
131. Baryshyan AL, Domigan LJ, Hunt B, Trimmer BA, Kaplan DL. Self-assembled insect muscle bioactuators with long term function under a range of environmental conditions. *RSC Adv* 2014; **4**: 39962–39968.
132. Akiyama Y, Sakuma T, Funakoshi K, Hoshino T, Iwabuchi K, Morishima K. Atmospheric-operable bioactuator powered by insect muscle packaged with medium. *Lab Chip* 2013; **13**: 4870–4880.
133. Guo SZ, Gosselin F, Guerin N, Lanouette AM, Heuzey MC, Therriault D. Solvent-cast three-dimensional printing of multifunctional microsystems. *Small* 2013; **9**: 4118–4122.
134. Miller JS, Stevens KR, Yang MT, Baker BM, Nguyen D-HT, Cohen DM et al. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nat Mater* 2012; **11**: 768–774.
135. Kolesky DB, Truby RL, Gladman AS, Busbee TA, Homan KA, Lewis JA. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv Mater* 2014; **26**: 3124–3130.
136. Peng H, Mankoff J, Hudson SE, McCann J. A layered fabric 3D printer for soft interactive objects. In: *Proceedings of the ACM CHI'15 Conference on Human Factors in Computing Systems: Design and 3D Object Fabrication*. Seoul, Korea, 2015, pp. 1789–1798.
137. Pei E, Shen J, Watling J. Direct 3D printing of polymers onto textiles: Experimental studies and applications. *Rapid Prototyp J* 2015; **21**: 556–571.
138. Sochol RD, Sweet E, Glick CC, Venkatesh S, Avetisyan A, Ekman KF et al. 3D printed microfluidic circuitry via multijet-based additive manufacturing. *Lab Chip* 2016; **16**: 668–678.
139. Wu GH, Hsu SH. Review: Polymeric-based 3D printing for tissue engineering. *J Med Biol Eng* 2015; **35**: 285–292.

140. Ahn D, Kweon JH, Kwon S, Song J, Lee S. Representation of surface roughness in fused deposition modeling. *J Mater Process Technol* 2009; **209**: 5593–5600.
141. Ahn D, Kweon JH, Choi J, Lee S. Quantification of surface roughness of parts processed by laminated object manufacturing. *J Mater Process Technol* 2012; **212**: 339–346.
142. Bhattacharjee N, Urrios A, Kang S, Folch A. The upcoming 3D-printing revolution in microfluidics. *Lab Chip* 2016; **16**: 1720–1742.
143. Billiet T, Vandenhaute M, Schelfhout J, Van Vlierberghe S, Dubruel P. A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials* 2012; **33**: 6020–6041.

Chapter 7 Bioprinting in otolaryngology and airway reconstruction

David A. Zopf and Glenn E. Green

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The specialty of otolaryngology-head and neck surgery comprises surgery of the craniofacial composition, ear, nose, throat, neck, and airway. Within this specialty, there are several clinical conditions that lack an ideal treatment. For these challenges, current techniques exhaust the demand and often fall short of the level of precision and customization that are necessary for satisfactory outcomes. Additive manufacturing and bioprinting provide paradigm-shifting potential for treatment options exceeding prior standard therapies.

In this chapter, we describe the current state of clinically challenging conditions that have set the stage for applications of additive manufacturing and bioprinting.

To date, application of additive manufacturing for airway and facial reconstruction can be generally grouped into (1) scaffold-based engineering guiding native tissues, (2) cell-seeded scaffolds, and (3) cell or hydrogel deposition printing.

7.1 Scaffold-based engineering for airway reconstruction

Scaffold-based engineering employs the precision provided by additive manufacturing techniques and provides a structural guide for native tissues. Advances in materials and device design have allowed for patient and disease specificity not only in the three-dimensional anatomic and structural sense but also in the fourth dimension by anticipating and planning for growth in, for instance, the infant and pediatric population.¹

Carvable graft materials and airway implants may be manually modified by their surgeon to match a patient's anatomy. In contrast, 3D-printed scaffolds have the

capacity to be specifically manufactured to the patient's unique anatomy at a sub-millimeter resolution, exceeding capabilities of standard manufactured devices and hand-carved reconstructions produced by the surgeon in the operating room. Immediately available patient-specific scaffolds would also truncate the duration of time required by the surgeon to customize those tissues and to drastically decrease associated risks of donor site harvesting and general anesthetic.

Tracheobronchomalacia (TBM) is pathologic dynamic collapse from weak airway structural support that can lead to cardiopulmonary arrest. With growth and maturation, infants are frequently able to overcome milder forms of the condition. When additional intervention is necessary, available treatments include positive pressure ventilatory support, tracheostomy, and further, oftentimes prolonged, positive pressure ventilatory support, and cardiovascular interventions such as aortopexy or intraluminal stenting. For severe TBM, all available options have high rates of failure and complications.^{2,3}

In 2013, our group at the University of Michigan, Ann Arbor, Michigan reported the first in human case of a patient-specific, computer-aided designed, laser-sintered airway splint.⁴ The device provided life-saving support for an infant with severe, life-threatening TBM and included one-year follow-up data. Follow-up for this first patient has exceeded five years and the splint has performed and resorbed as anticipated. A preclinical porcine model of TBM, the device demonstrated, increased survival compared to the untreated group.⁵

Subsequently, we reported our process of manufacturing 3D-printed personalized pediatric medical devices by adapting these novel processes to the rigors of Food and Drug Administration (FDA) device approval processes.⁶ In fact, these processes and collaborative efforts have served as the prototypic pathway for FDA approval of patient-specific, 3D-printed medical devices.⁷

The tracheobronchial airway splint is designed to resist anterior compression, allow flexion and extension, and open posteriorly with ease, thereby facilitating paediatric airway growth. This imparts four-dimensional design properties to the device. The splint is an open, bellowed cylindrical design. A 90° open angle allows placement of the device over the airway. External placement is intended to preserve the native mucociliary escalatory clearance and to avoid migratory and obstructive inflammatory issues seen with internal stenting. Bellow height and periodicity allow flexion of the device in the *z*-axis. Inner diameter, splint length, wall thickness, and number and spacing of suture holes can be customized to patient anatomy. Predesigned suture holes facilitate suture suspension of the airway wall to the splint support, thereby increasing the radius of the native airway and increasing the airflow as predicted by the Hagen–Poiseuille equation (Figure 7.1). The resorbable polycaprolactone (PCL) was selected to provide airway support for infants for approximately 24–36 months, followed by resorption and avoidance of the need for a procedure to remove the device.⁸

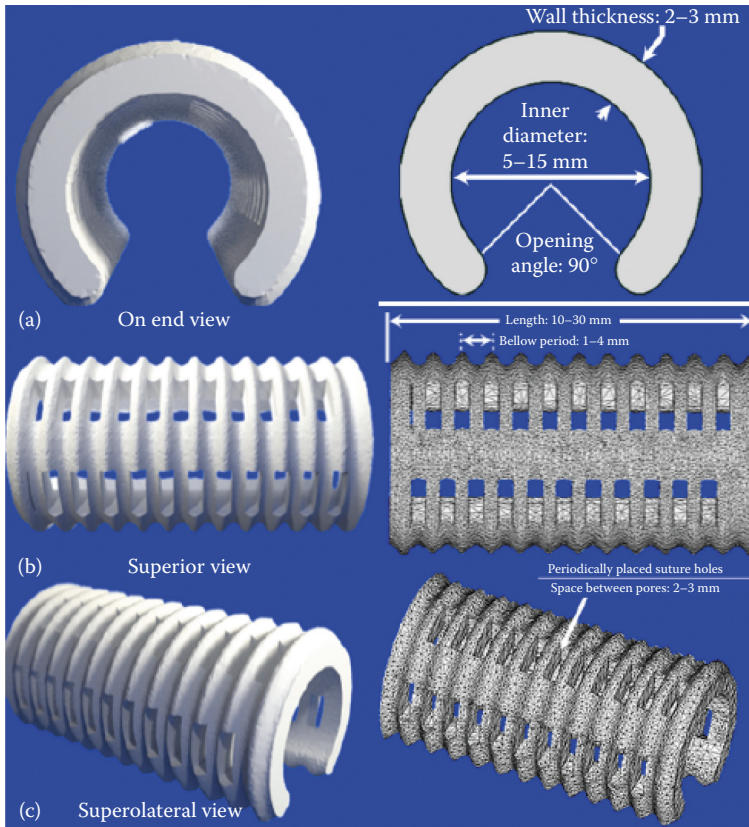


Figure 7.1 CAD renderings of tracheobronchial airway splint detailing several design targets. On end, coronal view (a), superior (b), and superolateral (c) views, which convey inner diameter, wall thickness, and opening angle; length and bellow period; and suture hole design placement, respectively.

Imaging of the patient's airway is performed using dynamic multidetector computed tomography (CT). The design parameters for the tracheobronchial splint were input into a proprietary MATLAB® (Mathworks) code to generate an output as a series of .TIFF image slices using Fourier series representation to generate the waves for bellow structures. The .TIFF image files were imported into Mimics and used to generate a surface .STL file of the splint from the image data using a variant of the marching cubes algorithm. The splint was then placed over the 3D model of the patient's airway for final design validation. Finite element analysis of each splint design is performed to ensure that the design met mechanical performance criteria noted below prior to manufacture. The verified .STL meshes of the splint designs are used to generate volume meshes with boundary conditions for simulating

mechanical testing (Altair Hypermesh, Altair Engineering). Both 8-nodal hexahedral and 10-nodal tetrahedral meshes are generated and tested for convergence.

The splint .STL is then imported to a Formiga P 100 System (EOS e-Manufacturing Solutions) and manufactured through a laser-sintering process from a blend of 96% CAPA 6501 PCL (Polyscience Inc) and 4% hydroxyapatite (Plasma Biotel Ltd.) flowing agent. PCL is cryogenically milled to a median particle size of 40–60 μm (Jet Pulverizer Co). Laser-sintering parameters are based on prior work by our group and include: laser power of 4 Watts, bed temperature of 50°C–56°C, laser scanning speed of 1000–1500 mm/s, and scan spacing of 0.15–0.20 mm (35–37). Splint models are orientated longitudinally along the z-axis during slicing. Postproduction processing involves air blasting followed by sonication in 70% ethanol for 30 minutes. The splints are peel packaged and undergo ethylene oxide sterilization at 49.0°C (Figure 7.2).

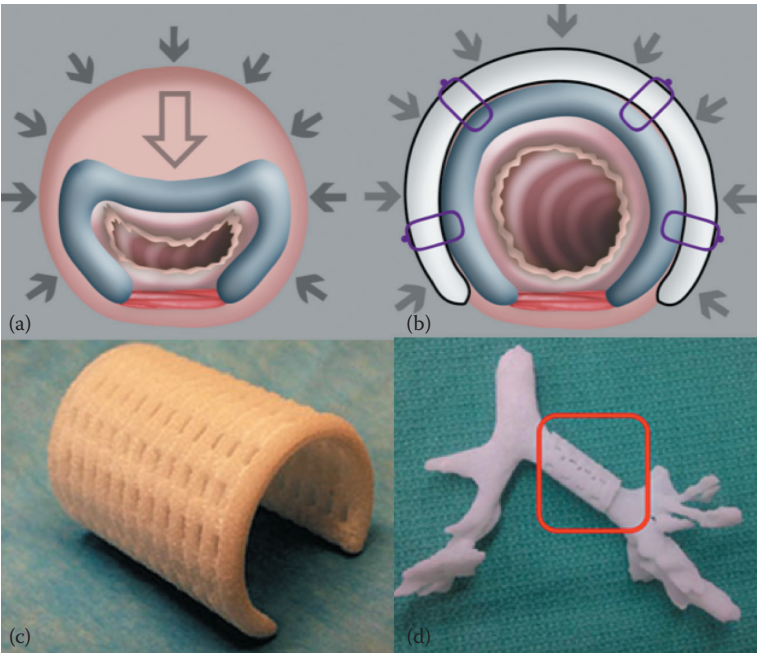


Figure 7.2 Airway collapse in severe tracheobronchomalacia can be accompanied by complete airway obstruction and life-threatening clinical condition. Compressive anatomic forces are depicted by arrows (a). The tracheobronchial airway splint is designed to ameliorate collapse by providing external rigid support to suture (purple) suspend the native airway open (b). A manufactured, patient-specific, 3D-printed, polycaprolactone tracheobronchial airway splint (c). Depiction of precise anatomic match and fit for patient-specific, 3D-printed, polycaprolactone tracheobronchial airway splint. Malacic collapsing left main stem bronchus region is framed in red (d).

Our group then demonstrated the use of additive manufacturing to provide a template for mature airway tissues. An adolescent woman with severe autism and thoracic scoliosis had developed acquired tracheomalacia due to compression between a high-riding innominate artery and thoracic spine within a narrowed thoracic inlet. Her disease severely progressed leading to cardiopulmonary arrests despite pharmacologic paralysis, mechanical ventilation, and continuous sedation for over 40 days. Previous treatment attempts included manubriectomy, aortopexy, and innominate artery reimplantation, though her severe, focal, compressive tracheomalacia was recalcitrant to these attempts. Discontinuation of care was recommended by her physicians. In this scenario, we elected to manufacture the splint using a nonresorbable material, knowing significant additional airway growth was not anticipated. For this scenario, polyetherketoneketone (PEKK) (OXPEKK; Oxford Performance Materials) was chosen. As in each of the pediatric cases, this young woman had resolution of her critical condition after management of her TBM with splinting.⁹

In early small-animal pilot studies, scaffold-based tissue engineering has been used to produce cartilaginous airway tissue. Goldstein et al. designed a rudimentary boat-shaped scaffold intended for use in an animal model of laryngotracheal reconstruction. The polylactic acid (PLA) grafts were printed using a simple MakerBot Replicator Desktop Printer with dimensions of 8 mm in length, 3 mm in width, and 1.2 mm in depth. Grafts were then seeded with rabbit tracheal chondrocytes in a type I collagen gel. Cartilage growth was demonstrated on *in vitro* and postvivo assessment.¹⁰

Adding layers of sophistication and complexity, Chang et al. reported fibrin/mesenchymal stem cell (MSC)-coated 3D-printed PCL scaffolds in the reconstruction of a partial tracheal defect in a rabbit model. 3D-printed scaffold fabrication was done with PCL (Sigma-Aldrich, St. Louis, MO, USA), as previously described (13). Polymer pellets were melted at 100°C–130°C in a heating cylinder. Halfpipe-shaped scaffolds (10 × 10 mm, diameter 10 mm) were made using the Bioplotter System (EnvisionTEC GmbH, Gladbeck, Germany). PCL was ejected through a heated nozzle, and the strand of PCL was plotted as layer-by-layer deposition on a stage. The nozzle size and distance between strands were 200 (nozzle size)/300 (distance between strands) with 27 G. The thickness of the PCL scaffold was 2 mm, chosen to approximate the thickness of a normal rabbit trachea, and the grid size which was made by horizontal and vertical strands of PCL was 0.5 × 0.5 mm.

The MSCs were harvested from rabbit and pelleted by centrifugation, then resuspended in a solution containing human-derived fibrinogen (9–18 mg/mL) (Mokam Research Center, Suwon, South Korea). MSCs suspension of 5 × 10⁶ cells/mL was then mixed homogeneously with 110 KIU/mL aprotinin (Mokam Research Center), 60 U/mL thrombin (1000 U/mg protein) (Sigma, St. Louis, MO, USA), 50 U fibrin stabilizing factor XIII, and 50 mM CaCl₂. The scaffolds were coated

with the fibrin-containing MSCs. The scaffolds, both pores and PCL strands, were completely coated with the fibrin-containing MSCs in a layer as thin as possible so as not to narrow the implant inner lumen.¹¹

Despite apparent migration in one of the four rabbits implanted, the MSC-seeded PCL tissue scaffolds appeared to adequately repair an anterior tracheal defect. Neocartilage and ciliated epithelium were reported with no respiratory distress reported. Follow up extended to 8 weeks.

It should be stated that as the complexity of the bioprinting methods increases, so does the complexity of regulatory hurdles. Our group encourages mindfulness with regulatory processes in developing design targets and using the simplest, necessary design, and necessary manufacturing technique. Complexity, in the form of cell source, growth factor inclusion, need for biologic tissue transport, and manufacturing processes, is likely to inversely impact near-future FDA approval for human clinical use.

7.2 Scaffold-based engineering for facial soft tissue reconstruction

The precision and reliable reproducibility for complex geometric architecture that additive manufacturing allows render a natural and potentially critical role for the future of facial reconstructive surgery. The facial composition includes several geometrically complex structures—such as the ear and nose—the undulations and contours of which are the most technically challenging to reconstruct and achieve desirable outcomes.

Reconstructing structures such as the external ear is indicated in several scenarios. The most common is when the ear structure is congenitally malformed—a condition called microtia, when the ear is smaller and dysplastic, or anotia when there is complete absence of external ear structure. The incidence of ear deformities is approximately 1% of live births, with severe microtia occurring in approximately 3 in 10,000. Acquired ear deformity can be seen in trauma or in cancer resections.

The current gold standard for ear reconstruction utilizes the patient's own autologous rib cartilage as foundational support for overlying soft tissue. These techniques demand the highest level of surgical and artistic ability as they rely on freehand carving of autologous cartilage, much like carving a sculpture.^{12,13} Recreating the undulating peaks and valleys demands in-depth experience and innate artistic ability. Even in the best of hands, outcomes can be inconsistent and undesirable. The amount of autologous tissue needed to carve an ear framework requires a large volume of a child's rib cartilage, delaying initiation of ear reconstruction until the approximate age of 7–10 years, and lengthening the window of psychosocial harm.¹⁴

There are several risks of this major reconstructive procedure including risk of pneumothorax and infection, severe donor site pain, discomfort, prolonged

operative time and anesthetic exposure, neurocognitive ramifications of such anesthetic exposures, increased hospitalizations, and prolonged recovery. Furthermore, ear reconstruction with autologous rib typically requires several procedures, adding a multiplier to these negative aspects of the procedure. Inconsistent results have led to low patient and family satisfaction in many surgeons' hands.¹⁵

Implantable high-density porous polyethylene, a permanent biocompatible polymer approved under the trade name MedPor, has been used for ear reconstruction.¹⁶ MedPor was approved for use by the FDA as a class II device through the 510k pathway. However, only a single MedPor construct is available to meet the needs of the wide range of pediatric and adult patients needing reconstruction.

Rates of framework fracture, exposure, extrusion, and infection of porous polyethylene are not transparently reported, though likely unacceptably high. These complications frequently require subsequent operations and anesthetic exposures with associated risks to address these complications. In addition, MedPor does not have the capacity for growth nor does it serve as a platform for cell seeding or tissue in growth.

Anaplastic prosthetic ear use is an additional option discussed with patients. Aesthetic outcomes can be excellent, however, patients and families rarely elect to pursue this pathway. Reasons for low interest in this option include limited durability and longevity paired with high cost of the prosthetic, need for multiple replacements particularly for the long projected lifespan of pediatric patients, and potential for the prosthesis to dislodge at inopportune and embarrassing times. Patients who have pursued an ear prosthetic have reported dissatisfaction for not identifying or recognizing the ear as a component of the patient's own self.

Tissue engineering is a promising alternative to rib grafts or synthetic constructs for the future of ear reconstruction. However, difficult obstacles facing tissue engineering and ear framework persist such as maintaining the intricate auricular geometry over time and avoiding mechanical contraction and distortion.^{17,18}

Vacanti et al. received notable coverage after first reporting implantation of an ear on the dorsum of rats in 1992. The exposed challenges for tissue engineering in ear reconstruction were not broadly highlighted by this seminal work. Vacanti's group found contraction of the scaffold to be a significant issue. Their ear scaffold manufacturing methods utilized clay-negative molds with PGA fill coated with PLA/PLGA. The several decade-length of time since this initial report and the lack of clinical translation in humans to date highlight the significant design and regulatory hurdles and illustrate the need of clinical feasibility as an initial primary design target.

Additive manufacturing recently emerged as a great potential for ear tissue engineering. Cai et al. were the first to describe the conceptual use of scaffold-based

engineering for ear tissue reconstruction. During seminal pilot work, material selection was far from ideal in this preliminary work. ABS (acrylonitrile butadiene styrene) scaffolds, a material that is not biocompatible, were manufactured through fused deposition modeling (FDM) (FDM 3000, Stratasys, Minnesota, USA). Scaffolds were then coated with fibronectin to promote cell adhesion and incubated with fibroblasts with and without keratinocytes.^{19,20}

The use of CAD/CAM to produce negative molds for scaffolds has been described. Manufacturing by Liu et al. was achieved by compressing PLA/PGA in negative ear-shaped molds,²¹ whereas Rieffel et al. utilized collagen as the fill material.²² Multipod photography data were converted to stereolithography (.STL) files using Studio 4.0 (Geomagic, Morrisville, NC) and imported into SolidWorks (Dassault Systems Corp, Waltham, MA). The image of the 3D ear was embedded into a virtual block, which was used to design a 7-part mold (SolidWorks). Each of the mold parts was printed out of acrylonitrile butadiene styrene (ABS) plastic using a Stratasys FDM 2000 3D printer (Eden Prairie, MN).

Notable cartilage growth was demonstrated by both groups. Framework distortion was noted in the former group; the latter group reports “[retention] of general contours” and relative maintenance of framework dimensions when comparing chondrocyte seeded to nonseeded scaffolds.

Our group was the first to propose patient-specific, 3D-printed, positive porous scaffold-based designs for ear reconstruction.²³ In addition we proposed a work flow aimed at feasible patient-specific clinical translation. We utilize a hierarchical image design for the patient-specific ear. The design was represented by a density distribution within a voxel format, similar to the way that 3D images are represented by density distributions within a voxel dataset. A separate voxel design dataset is created for the ear structure. This approach allows for heterogeneous structures created by designating different voxel densities in different physical locations. Various micropore structures may be designed into the scaffold, such as spherical or random porosities, using density distributions in voxel data structures with specially written MATLAB codes (The Mathworks, Natick, Massachusetts). These codes map the pore structure over a region that encompasses the final size of the anatomic region. The voxel structure is converted into a triangular surface .STL representation. The final scaffold design is created by mapping the porous architecture .STL file into the appropriate location of the anatomic dataset (also represented as an .STL file after conversion in the commercial software MIMICS [Materialise, Leuven, Belgium]).

Mapping of the porosity into the global patient-specific anatomic design using Boolean intersection operations of the custom-designed porous architecture .STL file and the anatomic region (auricle or nasal) .STL file is accomplished with MIMICS to create the final scaffold design.

The final design created by Boolean operations presents a significant manufacturing challenge. We^{24–27} use laser sintering of PCL in a similar fashion to that of the manufacturing of airway splints previously described (PCL, Polysciences, Warrington, Pennsylvania; PCL Preparation, Jet Pulverizer, Moorsetown, New Jersey).

A P100 laser sintering system (EOS North America, Novi, Michigan) is employed to fabricate patient-specific nose and ear scaffolds. The laser sintering process can fabricate feature sizes on the order of 700 μm (0.7 mm) (Figure 7.2).

Being similar in geometric complexity to ear reconstruction, nasal reconstruction, either partial or total, poses a significant challenge to the most experienced reconstructive surgeon. Total nasal reconstruction, in particular, requires significant effort and technical skill. In the best hands, unpredictable and undesirable outcomes are not uncommon. Nasal reconstruction is frequently necessary as a result of oncologic resections from skin malignancy, nasal trauma, or blast injuries are seen too often in our service men and women. Rarely, congenital malformation, such as arhinia, meets the indication for total nasal reconstruction, again posing a significant surgical reconstructive challenge, often requiring many complex, lengthy, and risky surgeries (Figure 7.3).

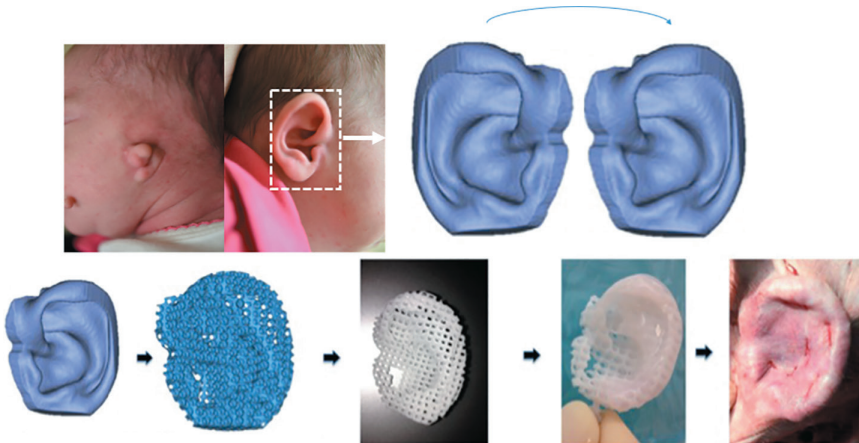


Figure 7.3 Proposed workflow for auricular tissue engineering utilizing clinically feasible bioprinting methods. An infant with left grade III microtia and a normal right ear would undergo capture of the normal right ear geometry with either DICOM imaging methods or multipod photography, followed by mirroring of the .STL, upper panel. The .STL then incorporated microporosity as described followed by laser sintering manufacturing of the positive scaffold. Chondrocyte embedded collagen-based hydrogen is then integrated and the scaffold is subcutaneously implanted.

Our group was also the first to propose a patient-specific, 3D-printed, porous scaffold-based design for nasal reconstruction.²³ Design and manufacturing methods are analogous to the production of ear scaffolds.

Our methods and craniofacial scaffolds have been tested in rodent and porcine animal models with robust demonstration of cartilage growth and short maintenance of scaffold dimensions (Figure 7.3). We have seeded our craniofacial scaffolds with primary auricular chondrocytes and in coculture environment with adipose-derived stem cells, both exhibiting robust cartilage growth. Long-term data are necessary prior to human use, however our proposed manufacturing methods are designed for feasible, near-future translation.

7.3 Bioprinting for facial soft tissue reconstruction

Bioprinting techniques and systems include (1) extrusion,²⁸ (2) jetting,²⁹ (3) laser-induced forward transfer (LIFT),³⁰ and (4) integrated tissue-organ printing (ITOP).³¹ Cell printing typically involves a hydrogel carrier, providing a viable, biocompatible environment and more predictable transfer methods. Each existing method has had limitations secondary to difficulty in manufacturing stable significant anatomic structures, limitations of structural stability, and flow rate limitations.

Of the methods listed, only the ITOP method has reported production of larger scale facial soft tissue constructs. Although ear geometries were successfully bioprinted, these constructs were approximately one half of the size of an average adult ear. Kang et al. described a multinozzle extrusion system producing composite scaffolds composed of internal PCL, gelatin/fibrinogen/hyaluronic acid/auricular chondrocyte hydrogel, and an external sacrificial Pluronic F-127 hydrogel.³¹ PCL is chosen for its previously stated property of stable structural support, prolonged degradation time of approximately two years, and its low melting temperature of 60°C, conducive to coprinting with cell containing hydrogels. The group used Pluronic F-127, despite poor biocompatibility, as added structural support. Gelatin was selected for its thermosensitive properties, solidifying below 25°C, liquefying above 37°C. Fibrinogen was chosen to encourage cell adhesion and proliferation. Hyaluronic acid and glycerol were utilized to facilitate flow and homogeneity. The materials are delivered via a three-axis stage, where the PCL nozzle included a heating unit allowing polymerization. CT imaging was used as the basis for CAD models, though presumably scaled down as scaffolds measured only $3.2 \times 1.6 \times 0.9$ cm.

Standard outcomes were reported including glycosaminoglycan content, histologic comparison to native auricular cartilage (H/E, Safranin O, Alcian Blue, and Collagen II), and biomechanical characteristics. Although gross appearance of the explanted ear scaffold at 1 month is provided, *in vivo* subcutaneous appearance *in situ* was not displayed. Nonetheless, this impressive report further details the ITOP method for manufacturing mandibular bone segments, calvarial bone, and skeletal muscle.

The potential that additive manufacturing and bioprinting have introduced to otolaryngology-head and neck surgery and airway reconstruction potentiates significant improvement in the possibilities for exceedingly challenging clinical scenarios with limited treatments and undesirable outcomes at present. In the case of the tracheobronchial splint, children in extremely critical clinical compromise and life-threatening condition were provided a specific medical device and procedure, designed for each individual, with life-saving results. Tissue-engineered scaffolds for facial reconstruction hold promise to give individuals their sense of wholeness or restore individuals to their original form and function in a manner that averts poor surgical outcome, mitigates surgical risk, and limits operative and anaesthetic time.

References

1. Tibbits S. 4D printing: Multi-material shape change. *Archit Design*. 2014; 84:116–121.
2. Murgu SD, Colt HG. Tracheobronchomalacia and excessive dynamic airway collapse. *Respirology*. 2006; 11(4):388–406.
3. Boogaard R, Huijsmans SH, Pijnenburg MW, Tiddens HA, de Jongste JC, Merkus PJ. Tracheomalacia and bronchomalacia in children: Incidence and patient characteristics. *Chest*. 2005; 128:3391–3397.
4. Zopf DA, Hollister SJ, Nelson ME, Ohye RG, Green GE. Bioresorbable three-dimensional printed airway splint. *New Engl J Med*. 2013; 368:2043–2045.
5. Zopf DA, Flanagan CL, Wheeler M, Hollister SJ, Green GE. Treatment of severe porcine tracheomalacia with a 3-dimensionally printed, bioresorbable, external airway splint. *JAMA Otolaryngol Head Neck Surg*. 2014; 140(1):66–71.
6. Hollister SJ, Flanagan CL, Zopf DA et al. Design control for clinical translation of 3D printed modular scaffolds. *Ann Biomed Eng*. 2015; 43(3):774–786.
7. FDA, Technical considerations for additive manufactured devices—Draft Guidance for Industry and Food and Drug Administration Staff (May 10, 2016), 81 Fed. Reg. 28876.
8. Morrison RJ, Hollister SJ, Niedner MF et al. Mitigation of tracheobronchomalacia with 3D-printed personalized medical devices in pediatric patients. *Sci Transl Med*. 2015; 7(285):285ra64.
9. Morrison RJ, Sengupta S, Flanagan CL et al. Treatment of severe acquired tracheomalacia with a patient-specific, 3D-printed, permanent tracheal splint. *JAMA Otolaryngol Head Neck Surg*. 2017; 143(5):523–525.

10. Goldstein TA, Smith BD, Zeltsman D, Grande D, Smith LP. Introducing a 3-dimensionally printed, tissue-engineered graft for airway reconstruction: A pilot study. *Otolaryngol Head Neck Surg.* 2015; 153(6): 1001–1006.
11. Chang JW, Park SA, Park J et al. Tissue-engineered tracheal reconstruction using three-dimensionally printed artificial tracheal graft: Preliminary report. *Artif Organs.* 2014; 38(6): 95–105.
12. Brent B. The correction of microtia with autogenous cartilage grafts, the classic deformity. *Plast Reconstr Surg.* 1980; 66:1–12.
13. Nagata S. A new method of total reconstruction of the auricle for microtia. *Plast Reconstr Surg.* 1993; 92:187–201.
14. Johns AL, Lucash RE, Im DD, Lewin SL. Pre and post-operative psychological functioning in younger and older children with microtia. *J Plast Reconstr Aesthet Surg.* 2015; 68(4):492–497.
15. Murabit A, Anzarut A, Kasrai L, Fisher D, Wilkes G. Teaching ear reconstruction using an alloplastic carving model. *J Craniofac Surg.* 2010; 21(6):1719–1721.
16. Romo T 3rd, Presti PM, Yalamanchili HR. Medpor alternative for microtia repair. *Facial Plast Surg Clin North Am.* 2006; 14:129–136.
17. Otto IA, Melchels FP, Zhao X, Randolph MA, Kon M, Breugem CC, Malda J. Auricular reconstruction using biofabrication-based tissue engineering strategies. *Biofabrication.* 2015; 7(3):032001.
18. Kamali P, Dean D, Skoracki R, Koolen PG, Paul MA, Ibrahim AM, Lin SJ. The current role of three-dimensional (3D) printing in plastic surgery. *Plast Reconstr Surg.* 2016; 137:1045–1055.
19. Pomerantseva I, Bichara DA, Tseng A et al. Ear-shaped stable auricular cartilage engineered from extensively expanded chondrocytes in an immunocompetent experimental animal model. *Tissue Eng Part A.* 2016; 22:197–207.
20. Cai H, Azangwe G, Shepherd, DET. Skin cell culture on an ear-shaped scaffold created by fused deposition modelling. *Biomed Mater Eng.* 2005; 15(5):375–380.
21. Liu Y, Zhang L, Zhou G et al. In vitro engineering of human ear-shaped cartilage assisted with CAD/CAM technology. *Biomaterials.* 2010; 31(8):2176–2183.
22. Reiffel AJ, Kafka C, Hernandez KA et al. High-fidelity tissue engineering of patient-specific auricles for reconstruction of pediatric microtia and other auricular deformities. *PLoS One.* 2013; 8(2):e56506.
23. Zopf DA, Mitsak AG, Flanagan CL, Wheeler M, Green GE, Hollister SJ. Computer aided-designed, 3-dimensionally printed porous tissue bioscaffolds for craniofacial soft tissue reconstruction. *Otolaryngol Head Neck Surg.* 2015; 152(1):57–62.
24. Williams JM, Adewunmi A, Schek RM et al. Bone tissue engineering using polycaprolactone scaffolds fabricated via selective laser sintering. *Biomaterials.* 2005; 26:4817–4827.
25. Partee B, Hollister SJ, Das S. Selective laser sintering process optimization for layered manufacturing of CAPA 6501 polycaprolactone bone tissue engineering scaffolds. *J Manuf Sci Eng.* 2006; 128:531–540.
26. Smith M, Flanagan CL, Kempainen JM et al. Computed tomography-based tissue engineered scaffolds in craniomaxillofacial surgery. *Int J Med Robot.* 2007; 3:207–216.
27. Mitsak AG, Kempainen JM, Harris M et al. Effect of polycaprolactone permeability on bone regeneration in vivo. *Tissue Eng.* 2011; 17:1831–1839.

28. Derby, B. Printing and prototyping of tissues and scaffolds. *Science*. 2012; 338:921–926.
29. Mironov V, Boland T, Trusk T. Organ printing: Computer-aided jet-based 3D tissue engineering. *Trends Biotechnol*. 2003; 21:157–161.
30. Ferris CJ, Gilmore KG, Wallace GG et al. Biofabrication: An overview of the approaches used for printing of living cells. *Appl Microbiol Biotechnol* 2013; 97:4243–4258.
31. Kang HW, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnol*. 2016; 34(3):312–319.



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Chapter 8 Bioprinting of human skin

Gaps, opportunities, and future directions

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8.1 Human skin: A complex integument

Skin is one of the most investigated tissues for *in vitro* reconstruction, possibly, due to the relatively simple, hierarchical, and layer-by-layer structure formed by the dermal compartment (papillary and reticular dermis) and the multiple epidermal

layers (*stratum basale*, *spinosum*, *granulosum* and *stratum corneum*) that can be reproduced *in vitro* with relative ease (Eckert and Rorke 1989; Reijnders et al. 2015; Zhang and Michniak-Kohn 2012; Moulin et al. 2011; Harper and Grove 1979). The reconstructed skin models typically contain fibroblasts embedded in a collagenous matrix to simulate the dermis, and augmented with a layer of keratinocytes atop the dermis to simulate the epidermis. When exposed to optimal and biologically/physiologically relevant conditions, this assembly can differentiate and mature to form a stratified multilayered structure that resembles human skin. Nonetheless, this uniform and quite predictable structure recreated *in vitro* does not recapitulate the complexity of the skin. *In vivo*, skin presents a diverse population of cells, and a complex and heterogeneous distribution of proteins and growth factors that cannot be adequately represented in the simplified models fabricated in the laboratory (Panteleyev et al. 2001). Furthermore, many of these cells are organized and compartmentalized in distinct structures within the skin, such as vasculature, and adnexal structures (for e.g., hair follicles, sweat glands, and sebaceous glands), which confers an even higher level of complexity to this organ.

8.1.1 Diversity of cell populations

The human skin contains multiple cell populations beyond keratinocytes, fibroblasts, and the cells from the appendages, and whose functions are fundamental for maintaining proper skin homeostasis. The Langerhans cells, from the *stratum spinosum*, and the dermal dendritic cells are among the cells responsible for initiating the immune response against external pathogens (Metcalfe and Ferguson 2007; Bechetoille et al. 2007; Zhang and Michniak-Kohn 2012). Perception of touch, temperature, and pressure in the skin can be attributed to Merkel–Ranvier cells within the *stratum basale* (Metcalfe and Ferguson 2007; Bechetoille et al. 2007; Zhang and Michniak-Kohn 2012). Melanocytes, another important component of the epidermis, are also evenly distributed among the proliferative keratinocytes of the *stratum basale* (Velasquillo et al. 2013). The melanin produced by these cells is transferred to the surrounding keratinocytes, enhancing skin protection against UV light (melanin is a known broadband UV absorbent) among other beneficial effects (Brenner and Hearing 2008; Yu 2002). Functionalization of skin models using nerve cells could lead to the recovery of tactile perception at grafted sites. It has been shown that Schwann cells encapsulated in the dermal compartment of a reconstructed skin model can promote neurite migration *in vitro* and functional recovery of nerves in the graft *in vivo* (Blais et al. 2009). The inclusion of immune cells in the different compartments of the reconstructed skin creates what is known as immunocompetent skin models. These models are an important tool in the industry for assessing skin sensitization potential of new drugs and chemicals (Chau et al. 2013).

The incorporation of additional cells, beyond keratinocytes and fibroblasts in reconstructed skin models will have an important impact both in regenerative medicine and in diversification of *in vitro* models available for safety assessment of chemical

compounds. The inclusion of these cells in a meaningful and relevant way can be facilitated by 3D bioprinting, a technology which can precisely place these cells at the appropriate skin depth within the dermal matrix or at the basal membrane.

8.1.2 Microvasculature

The microvasculature of skin forms a complex three-dimensional (3D) structure organized in two horizontal plexuses connected by ascending arterioles and descending venules (Yen and Braverman 1976; Braverman and Keh-Yen 1981; Braverman and Yen 1977; Braverman 2000). In the upper plexus, approximately 1–2 μm below the epidermis, arterial capillaries give rise to the dermal papillary loops. This is the site of primary exchange of oxygen and nutrients. In addition, ascending arterioles rising from the lower plexus, situated at the dermal subcutaneous junction, branch between the two plexuses to feed dermal appendages. The arterioles and venules found in the papillary dermis vary from 17 to 26 μm in diameter. Endothelial cells are surrounded by one or two layers of smooth muscle cells or pericytes and the vascular wall is mainly composed of a basement membrane. In contrast, arterioles and venules at the lower plexus are typically 50 μm in diameter with walls 10–16 μm thick. Endothelial cells are surrounded by 4 or 5 layers of smooth muscle cells or pericytes (Braverman and Sibley 1990; Braverman and Keh-Yen 1981). Moreover, collagen fibers also present different patterns in the two plexuses. At the lower plexus, collagen fibers can be found as bundles of 50–100 fibrils oriented along the vascular wall, whereas at the superficial plexus individual collagen fibrils are diffusely distributed in the peripheral zone of the vascular wall (Braverman 1997).

Abnormalities in skin microvascular organization are often associated with several pathological conditions of skin. In psoriatic lesions, for example, the hyperproliferative and parakeratinized epidermis are associated with an increased density of capillaries. The capillary loops, which are typically found in normal skin as single and arterial loops change to highly coiled, dilated, and venous loops in psoriatic lesions (Braverman and Sibley 1982; Braverman 1989). Cutaneous blood flow in patients with active psoriasis has been found to be 10 times higher than in healthy individuals (Klemp 1987). Capturing the ultrastructure and organization of the cutaneous microcirculation in both, normal physiology and disease states, remains a challenge in the development of improved skin equivalents. The incorporation of microvasculature that can structurally and functionally mimic *in vivo* responses may lead to better integration of engineered skin grafts with, and within, the host tissue as well as lead to the development of improved skin disease models.

8.1.3 Adnexal structures

Sweat glands: Sweat glands are primary functional units responsible for physical thermoregulation and hydration through the release of water and salt-based solutions (sweat) across the skin (Lu and Fuchs 2014).

There are two types of sweat glands in the skin: eccrine, which present ducts opening to the outside surface of the skin, and the apocrine, which have openings directly into the hair follicle (Lu and Fuchs 2014; Velasquillo et al. 2013). The lack of sweat glands in the reconstructed or artificial skin grafts used in the treatment of burn victims with extensive wounds in the body, can later lead to a life of skin thermoregulation impairment and excessive dryness of the transplanted skin area (Huang et al. 2010; Moulin et al. 2011).

Hair follicles and sebaceous glands: The pilosebaceous units, comprising hair follicles and sebaceous glands, are complex structures formed by about 15 types of cells of epithelial and mesenchymal origins distributed in concentric layers (Bernard 2017; Niemann and Horsley 2012). At early stages of development of the human body, the interaction between epithelial and mesenchymal cells allows the formation of this particular skin appendage (Higgins et al. 2013; Niemann and Horsley 2012). Throughout the life-cycle development, different stem cell populations within the skin support the cyclic regeneration of the hair follicles and sebaceous glands (Higgins et al. 2013; Niemann and Horsley 2012). The pilosebaceous unit is also connected to the apocrine sweat gland, the arrector pili muscle, the underlying skin vasculature, and nerve cells (Yano et al. 2001; Velasquillo et al. 2013; Bernard 2017). The complexity of this structure confers to this appendage the status of a *miniorgan* (Schmidt-Ullrich and Paus 2005; Bernard 2017). One of the main motivations for incorporating hair follicles in skin models is the presence of a stem cell population in the bulge region (Higgins and Christiano 2014; Balana et al. 2015). These cells have an important role not only in hair follicle renewal but they can also migrate to damaged skin sites to support epidermal regeneration, which can potentially improve integration of grafts with the host (Chunmeng and Tianmin 2004; Ito et al. 2005; Velasquillo et al. 2013). In addition, transplantation of hair follicle units generated *in vitro* could be applied for the treatment of diseases that lead to hair loss, such as androgenetic alopecia, skin depigmentation, and localized vitiligo (Pan et al. 2013; Chouhan et al. 2013; Yu 2002).

8.1.4 Unmet needs

The creation of more complex skin models, containing multiple cell types, vasculature, hair follicles, and sweat and sebaceous glands is significant for different applications. In regenerative medicine, there is a critical need for skin grafts that present better esthetic and functional recapitulation of native skin aiming at complete integration of the graft onto, and into, the wounded tissue. This also applies to grafts developed as a clinically superior alternative for the treatment of skin diseases (Velasquillo et al. 2013; Moulin et al. 2011). In the field

of drug and cosmetic screening, the motivation lies in the need for skin models that can result in better translation between the *in vitro* and *in vivo* responses (Vijayavenkataraman et al. 2016). In the subsequent sections, a review of current technologies for the incorporation of vasculature, adnexal structures, multiple cell populations, and a more representative matrix microenvironment in skin models is presented, with a specific focus on the tissue-engineering (TE) challenges, followed by the advances and opportunities afforded by 3D bioprinting.

8.2 Contemporary skin models: Gaps and limitations

8.2.1 Engineering grafts for regenerative medicine

In the past four decades, a variety of acellular and cellular matrices have been developed to promote skin wound healing, such as Alloderm™, Biobrane™, Dermagraft™, and Apligraf™ (please see reviews by Lazic and Falanga 2011 and Brohem et al. 2011 for further details). Although it was originally thought that cultured allografts had the potential to be used for skin replacement, it is now known that these do not integrate efficiently with the host skin. Instead, these artificial grafts act as pharmacological agents that stimulate wound healing by inducing reepithelization from the dormant wound edges, a process known as the edge effect (Kirsner et al. 1996). Moreover, cultured allografts work to provide immediate wound coverage from injuries and infections; release of healing growth factors (for e.g., TGF- α and β , GM-CSF, β FGF, PDGF, TNF- α) and cytokines (for e.g., IL-1, 3, 6, and 8) (Maas-Szabowski et al. 2003; Lim et al. 2009; Uchi et al. 2000); and biosynthesis of new matrix components (Boehnke et al. 2007). Phillips et al. (2002) have shown that allogeneic cells were rarely detected in patients with venous ulcers treated with Apligraf™ at 2 months after grafting. In agreement with this observation, Griffiths et al. (2004) demonstrated that Apligraf™ DNA does not persist beyond 4 weeks in the host skin. The relatively short clinical persistence of Apligraf™ and other cultured allografts can be a disadvantage, particularly in the treatment of chronic wounds, which require more than 4 weeks to heal. Since these grafts do not integrate with the host skin and are only temporary, patients often need multiple rounds of treatment until reepithelization is achieved. This makes the use of cultured allografts expensive, burdensome, time-consuming, and with suboptimal clinical outcomes.

One of the reasons cultured allografts cannot integrate with the host skin is the lack of a functional vasculature. Skin microvasculature not only promotes graft survival by supplying skin cells with oxygen and nutrients but also modulates inflammation and immune cell migration to the wound site (Pasparakis et al. 2014). Moreover, the presence of a vascularized bed has been shown to promote skin engraftment through microvascular connections between graft and recipient capillaries that surround the wound region (Gibot et al. 2010; Tremblay et al. 2005).

Since the early studies on the physiology of graft acceptance in the late nineteenth century by Bert (1865) and Thiersch (1869), there has been an ongoing debate between two opposing theories regarding the origin of skin graft revascularization: is it achieved through graft's vessel growth and connection with the host tissue, or is the new vascular supply originating by capillary invasion from the host bed? In 1865, pioneering studies by Bert (1865) demonstrated direct vascular connections between recipient and graft vessels as early as 18 hours after grafting. These findings founded the theory of anastomosis. Observations by Converse et al. (1975) on vascular changes in skin autografts in mice showed that blood flow was recovered 48 hours after grafting. This short time interval between grafting and blood perfusion reinforced Bert's anastomosis theory. Supporting the alternative theory, Garré (1889) reported evidence that graft vessels degenerated soon after engraftment, and therefore, new vascular ingrowth from the recipient bed had to occur. Despite many years of trying to understand the origin of skin graft revascularization, it was not until recently that it became possible to answer some of the most controversial questions on the mechanisms of graft acceptance. The development of improved imaging techniques and animal models provided researchers with new tools to understand these mechanisms. Using a *tie2/lacZ* transgenic mouse, Capla et al. demonstrated that skin graft revascularization occurs through recipient vascular ingrowth and simultaneous donor vascular regression (Capla et al. 2006). Recipient-derived endothelial cells use the donor vascular framework as a guiding conduit for vascular ingrowth. This dynamic process of regression and ingrowth events results in quick connection between the capillaries of the host and the graft. These findings suggest that successful skin revascularization is greatly dependent on a critical and short window of time in which anastomosis needs to occur to reestablish blood flow and oxygenation to the wound site. The presence of a preexisting vascular network accelerates this process facilitating the successful acceptance of the graft. In contrast, nonprevascularized grafts rely on a neovascularization process, which can take 3 weeks until blood flow is restored (Tsur et al. 1980). Failure in the reestablishment of the supply of oxygen and nutrients to the wound site often leads to graft rejection.

The development of new strategies to promote the graft–host angiogenic cross-talk that allows anastomosis and vascular integration of skin grafts is crucial to overcome the current limitations of skin graft acceptance. Approaches such as the *in vitro* generation of capillary-like networks embedded in engineered skin constructs before transplantation can not only yield faster integration within the host tissue but can also promote long-term skin homeostasis *in vivo*.

8.2.2 Developing disease models

For many years, researchers have been trying to gain better understanding of the biological mechanisms involved in human skin diseases (please see review by Mathes et al. 2014). Although significant advances have been made in creating

better animal models, many of these still fail in reflecting the human situation. This challenge has boosted efforts among biologists and engineers to create *in vitro* 3D models containing human cells that recapitulate the pathophysiology of the disease states in skin. Such alternative systems not only provide insights into the origin of the disease and its progression but can also be used as drug discovery platforms as they allow for high-throughput screening of topical agents and the development of improved diagnostic and prognostic approaches.

Psoriasis: Psoriasis is a chronic autoimmune disease that causes skin cells to accumulate rapidly on the surface of the skin, which creates red and itchy patches that may vary in size and severity. This building-up of skin cells results from the interaction between genetic and environmental factors that cause the overproduction of cytokines by T cells (Cai et al. 2012). This increase in cytokine production stimulates keratinocyte proliferation and the expression of adhesion molecules in the skin microvasculature to facilitate the infiltration of inflammatory cells into psoriatic skin to maintain high levels of cytokine production (Lee et al. 1994). Psoriasis was one of the first *in vitro* models to be developed in the context of skin pathology. In 1990, Krueger and Jorgensen developed a model composed of healthy or diseased dermal fibroblasts, and healthy keratinocytes separated by a porous membrane. However, this separation introduced a physical barrier that limited important interactions between the keratinocytes and fibroblasts. A more advanced skin model was later developed by van den Bogaard et al. with the inclusion of immune cells (van den Bogaard et al. 2012). The model was generated by prestimulated CD4⁺ cells from donor blood to facilitate Th1, Th2, and Th17 responses. These cells were then loaded on a membrane and covered with decellularized dermis. The authors demonstrated transdermal migration of the CD4⁺ cells toward the epidermis. The interaction between immune cells and keratinocytes in the epidermis increased the expression of biomarkers of inflammation (DEFB4, LCE3A, KRT16, and S1007), as well the expression of proinflammatory cytokines (IL6, IL8, and IL23). However, the levels of proinflammatory cytokines were still 40 times lower than that of psoriatic skin lesions indicating a less-than-sufficient representation of disease states, possibly due to the lack of the vascular compartment.

Melanoma: Cutaneous malignant melanomas are highly invasive and can easily metastasize to distant sites in the body. In the past decade, researchers have been trying to create appropriate *in vitro* models that can mimic human melanoma progression to address questions about the molecular mechanisms of melanoma invasion (please see review by Beaumont et al. 2013 for further details). Melanoma progression is characterized by three phases: (1) radial growth phase (RGP), vertical growth

phase (VGP), and metastatic melanoma (Miller and Mihm 2006). During RGP, melanoma cells grow as individual cells or small clusters along the basement membrane, whereas VGP melanoma cells are more aggressive as they gain the ability to invade the dermis by breaking through the basement membrane. Metastatic melanoma cells can invade both epidermis and dermis, reach vascular or lymphatic vessels, and subsequently spread to distant sites. Current models of melanoma attempt to recapitulate this multiphasic nature of melanoma progression through the integration of RGP, VGP, and metastatic melanoma cells in skin constructs. Meier et al. have successfully demonstrated that *in vitro* response of these cells in a 3D skin model accurately reflects the responses observed *in vivo*: RGP melanoma cells grew but could not penetrate the basement membrane of the epidermis; VGP melanoma cells invaded into the dermis; and metastatic melanoma cells rapidly proliferated in the dermis (Meier et al. 2000). In an attempt to create a more complex and relevant model of metastatic melanoma progression, Gibot et al. (2013) incorporated a microvasculature network within a reconstructed skin substitute to study the mechanisms of tumor angiogenesis. The presence of microvasculature within this model allowed the authors to demonstrate a correlation between high VEGF production by melanoma metastatic cells and migration of endothelial cells toward these cells. Moreover, the authors state "... the need to incorporate other structures such as lymphatic networks in melanoma models, since metastasis to the regional lymph nodes is one of the most important indicators of tumor aggressiveness, especially in melanomas" (Gibot et al. 2013).

As more becomes known about skin function, it also becomes clear that current skin models comprising of fibroblasts and keratinocytes alone lack the complexity of native human skin. Capturing the dynamic cross-talk between multiple cell types and structures in skin models is crucial and necessary to recapitulate the physiology of healthy and disease states.

8.2.3 Efficacy models for drug and formulation screening

Many *in vitro* skin substitutes such as EpiSkin™, skinEthic™, Epiderm™, and GraftSkin™ have received regulatory approvals in Europe and United States for testing skin corrosion, acute skin irritation, and phototoxicity (Botham et al. 1998; Netzlaff et al. 2005). However, since these models lack rete ridges, vasculature, and other appendages such as hair follicles, they are limited in the detection of adverse drug reactions and prediction of percutaneous absorption across native human skin. Moreover, because these substitutes do not fully recapitulate the normal barrier function of human skin, none of them is currently approved for testing skin absorption. Skin equivalents have been shown to exhibit 10-to-30 times

higher permeability of topical agents across the *stratum corneum* when compared to native skin (Perkins et al. 1999). This can result in an overestimation of systemic exposure to irritants due to their higher penetration rates. Due to these limitations of the current models, many percutaneous permeation studies still rely on the use of human cadaveric skin and animal skin obtained from pigs, mice, and rabbits. These models have been extensively used to investigate the molecular mechanism responsible for the development of skin diseases and in the discovery of new drug candidates (Jung and Maibach 2015). However, with increasing awareness for animal protection and regulation of the use of animals for the testing of cosmetics, companies are now increasing their efforts in developing *in vitro* skin models with better barrier function.

8.3 3D bioprinting: Advances and opportunities

The complexity of skin microvasculature and other 3D structures that are present in human skin are difficult to replicate *in vitro* through simple manual fabrication methods. 3D bioprinting is a flexible tool that allows precise deposition and patterning of different cell populations and biomaterials to create accurate geometries in three dimensions that mimic anatomical and biological structures such as tissues and organs (Munoz-Abraham et al. 2016; Knowlton et al. 2016; Murphy and Atala 2014).

8.3.1 Incorporating diversity of cell populations

Human skin is a rich repository of multiple cell populations, including immune cells such as Langerhans cells, and cells responsible for various phototypes as examples. Integrating Langerhans cells in reconstructed models is specially challenging because these cells cannot be easily isolated and expanded *in vitro* (Regnier et al. 1998). As an interesting alternative, cord blood-derived and CD34 + progenitor cells from the peripheral blood can be used to generate precursor dendritic cells (Dezutter-Dambuyant et al. 2006; Regnier et al. 1998). This population of hematopoietic-derived precursor dendritic cells can give rise to Langerhans cells in the epidermis, when cocultured with keratinocytes, or dermal dendritic cells, when encapsulated in the dermal compartment with the fibroblasts (Regnier et al. 1998; Dezutter-Dambuyant et al. 2006). This example demonstrates the importance of precisely localizing cells within the different skin layers, which can be easily achieved with precision on the order of micrometers using 3D bioprinting. *In vitro*, the appropriate patterning of melanocytes and keratinocytes, which can be achieved with the use of 3D bioprinting, would allow the development of skin grafts representing the diverse skin phototypes (Ng et al. 2016; Brohem et al. 2011; Metcalfe and Ferguson 2007). The skin phototypes (I–V), which reflects to some extent the skin color, are based on the classification of sunburns and tanning effects of the different skin types after sun exposure

(Fitzpatrick 1988). Skin grafts mimicking these different phototypes are currently not available (Metcalfé and Ferguson 2007). Furthermore, it has been suggested that 3D bioprinting could be used for seeding the melanocytes, *in situ*, at specific positions, along with the necessary growth factors, as a treatment option for diseases such as vitiligo or even to reduce the depigmentation during scarring process (Velasquillo et al. 2013).

8.3.2 Incorporating vasculature

Multiple approaches using 3D-bioprinting technology have shown to successfully generate perfusable capillary-like structures *in vitro* (please see reviews by Hoch et al. 2014 and Frueh et al. 2016 for further details). These studies have not yet shown integration of the vascularized constructs in the skin context. However, a few research groups have recently demonstrated the combination of both tissue components into a single vascularized skin model (Bibb et al. 2016; Abaci et al. 2016). Abaci et al. (2016) have successfully generated an *in vitro* skin model embedded with a 3D-bioprinted and perfusable vascular network using both primary and induced pluripotent stem cell (iPSC)-derived endothelial cells. The authors demonstrated in mice that blood flow was restored to the vascularized skin constructs 14 days after grafting through ingrowth of the host vessels that perfectly aligned with the patterns of the graft vessels. After gel contraction, the final size of the printed vessels ranged from 100–250 μm . Although the authors could create smaller channels with their platform, the lowest diameter achieved was of 80 μm . This size is significantly larger than the capillaries in the skin microvasculature: <26 μm at the superficial horizontal plexus and <50 μm at the dermal–subcutaneous plexus. In addition, a fully printed vascularized skin construct has yet to be realized. The development of a full-thickness vascularized 3D-printed skin model is not only important for the success of permanent engraftment but also for disease modeling of inflammatory conditions in systems such as organ-on-a-chip as it provides a platform for physiological integration with other organ systems.

8.3.3 Incorporating adnexal structures

The first attempt to include sweat glands in a human-reconstructed skin model was made in 2010 by Huang et al. (2010). They successfully showed that gelatin microspheres containing sweat gland cells and epidermal growth factor, when injected in a full-thickness skin model, could differentiate in structures similar to sweat glands. Furthermore, these constructs, when grafted on a wound in mice, improved tissue integration compared to control condition, demonstrating the importance of this skin appendage for tissue regeneration (Huang et al. 2010). Recently, the same group employed a 3D-bioprinting platform as a tool to promote differentiation and regeneration of sweat gland cells (Huang et al. 2016;

Liu et al. 2016). They printed a cell-laden, 3D extracellular matrix (3D-ECM) using gelatin and alginate hydrogels containing different growth factors (Huang et al. 2016). This scaffold, specifically defined spatial patterning of growth factors within the 3D-ECM, created an environment capable of inducing differentiation of epidermal progenitor cells into sweat gland cells (Huang et al. 2016). In addition, the matrix-induced sweat gland regeneration when it was directly printed on burn wounds in mice (Huang et al. 2016). Further experiments demonstrated the effect of the pore sizes and controlled growth factor release from the 3D-ECM, both guided by printing parameters, in the differentiation and self-organization of sweat gland cells (Liu et al. 2016). With this work, the group has demonstrated the advantages of using 3D bioprinting for precisely controlling scaffold structure aiming at the improvement in differentiation of sweat gland cells *in vitro*. The 3D-bioprinting technology allows not only the creation of a scaffold with precise and a reproducible architecture but also the simultaneous incorporation of multiple cells, with high cell viability, and matrix molecules during the fabrication of biological structures (Huang et al. 2016; Liu et al. 2016). Furthermore, the *in situ* 3D bioprinting of the 3D-ECM could be used as a way of delivering cells to patients with large burn areas more efficiently (Huang et al. 2016).

Scientists have been studying hair follicle development for more than three decades. Different approaches have been investigated for the regeneration of hair follicles *in vivo* and *in vitro*. One of the successful methods developed so far is known as the *organ germ method*. In this approach, epithelial and mesenchymal cells are injected in a hydrogel droplet and, following initial self-rearrangement, lead to the formation of the early *organ germs* (Toyoshima et al. 2012). After *in vitro* incubation, these structures are transferred to the subcutaneous region of mouse skin where they have shown to be able to reconstitute whisker fibers or human hair fibers depending on the origin of the cells (Nakao et al. 2007; Toyoshima et al. 2012). In a different approach, a skin model constituted by a dermal compartment containing dermal papilla cells as dissociated or self-assembled spheroids and an epidermal layer with primary keratinocytes was shown to generate hair follicles after a few weeks of engraftment on the back of a mouse model (Thangapazham et al. 2014).

Several of the successful studies in hair follicle neogenesis have employed mouse cells or a coculture of human and mouse cells (Kang et al. 2012; Takagi et al. 2016; Toyoshima et al. 2012). Nonetheless, the potential of human cells to generate hair follicles has been demonstrated by several research groups, but these protocols are not sufficient for production of human hair follicle units in large scale and in a reproducible manner (Balana et al. 2015; Thangapazham et al. 2014; Toyoshima et al. 2012). In contrast to mouse cells, human dermal papilla cells lose their inductive potential to form hair follicles during initial expansion phase in the monolayer culture (Lin et al. 2016; Ng et al. 2016; Higgins et al. 2013). Another key difference between the two species is that the human dermal papilla cells do not go through self-aggregation *in vitro* to the same extent as

the mouse cells (Higgins et al. 2013). As an attempt to overcome the challenges faced with 2D cultures, spheroid-based methods have been employed aiming at the restoration of the inductive capacity of human dermal papilla cells (Miao et al. 2014; Higgins et al. 2013; Lin et al. 2016). Higgins et al. (2013) demonstrated that human dermal papilla cells cultured as 3D spheroids restore part of their inductive capacity. In addition, they can generate hair follicle fibers following the transplantation into intact human skin grafted on the back of mice models (Higgins et al. 2013). This need for a specific 3D environment could be clearly addressed by the 3D-bioprinting technology, which, as previously stated, allows the arrangement of scaffolds and cells in specific patterns.

Focusing on the 3D structure of the hair follicle, Pan et al. (2013), using soft lithography, created an array of microwells that resembled the architecture of this skin appendage. When seeded with dermal and epidermal cells, the compartmentalized microwells supported the cell survival, and maintained the organization of cells demonstrating its potential as a method for generating follicle-like structures (Pan et al. 2013).

Based on prior studies in hair follicle neogenesis research, it is clear that a key step for successfully bioengineering human hair *in vitro* is the spatial distribution and compartmentalization of the epithelial and mesenchymal cells (Ohyama and Veraitch 2013; Mahjour et al. 2012; Balana et al. 2015). In spheroid cultures or in the organ germ method, epithelial and mesenchymal cells are tightly seeded in a hydrogel and it is their capacity for self-arrangement that leads to hair-follicle formation (Nakao et al. 2007; Toyoshima et al. 2012; Higgins et al. 2013). In a different approach, Pan et al. (2013) recreated the 3D architecture of the hair follicle using a prefabricated mold, which they hypothesize that, when seeded with appropriate cells, could lead to the formation of hair follicle-like structures.

With the advances in 3D-bioprinting technologies, the 3D microenvironment of the skin can be reproduced with higher precision: cells can be placed at defined positions within the scaffold/matrix, which is itself of a defined composition and structure. Applied to hair-follicle reconstruction, this concept facilitates the precise seeding of the epithelial and mesenchymal cells embedded within the extracellular matrix (ECM) to recreate the complex pilosebaceous unit.

8.4 Emerging concepts: Optimized bioinks

3D printers developed in the early 1980s have evolved into platforms considerably more efficient, sophisticated, and widely accessible (Vermeulen et al. 2017). These systems have been adapted for printing structures such as tissues and organs using biological inks containing proteins, glycosaminoglycans, growth factors, signaling molecules, and cells (Ouyang et al. 2016). The composition of the bioinks depends

primarily on the biological structure that one wishes to print, and the printing platform employed. The main constituents of the bioinks typically employed in 3D bioprinting are cells, growth factors, and structural components, such as ECM molecules and natural or synthetic hydrogels. Besides the mechanical and structural properties, the bioinks are necessary for providing the physical microenvironment and biochemical cues to the cells during the printing process, and subsequent tissue maturation.

8.4.1 Optimizing bioinks for platforms

A key step for successfully printing tissues and organs is to design optimized bioinks that satisfy the corresponding biological, chemical, and physical niche (Malda and Groll 2016; Ouyang et al. 2016). Along these lines, the specific mode of material deposition and subsequent phase-transition (for e.g. droplet/inkjet, extrusion, light or temperature-based curing, or microvalve-based pneumatic systems) is one of the first factors that needs to be considered to accommodate the material properties for each platform (Chimene et al. 2016; Holzl et al. 2016; Murphy and Atala 2014; Vermeulen et al. 2017). For example, the viscosity of bioinks feasible for extrusion-based bioprinters is higher than that for inkjet models (Chimene et al. 2016). Another important aspect of some bioinks is the gel-to-solid phase transitioning process required for hydrogels. For example, the gelation rate affects the structural integrity of the printed shape and the cross-linking method can affect the cell viability (Chimene et al. 2016; Ouyang et al. 2016). Finally, the biological properties of the bioink also need to be considered. For example, bioinks containing signaling molecules such as growth factors or binding sequences can promote the proliferation, migration, and differentiation of cells, as well as the subsequent remodeling of the matrix (Chimene et al. 2016; Ouyang et al. 2016). The bioinks should, therefore, have a good balance between the ideal mechanical properties and the desired biological characteristics (Chimene et al. 2016; Ouyang et al. 2016). This design process where biological and mechanical properties of a bioink must be taken into account has been referred to as the *biofabrication window* by Chimene et al. (2016).

8.4.2 Optimizing bioinks for scaffolds

A review of the literature reveals that several approaches have been used to successfully reconstruct human skin *in vitro*. Typically, keratinocytes are directly seeded on top of a dermal scaffold composed of synthetic substrates or matrix-derived proteins, such as type I collagen populated by fibroblasts. Nonetheless, as mentioned in [Section 8.2 Contemporary skin models: Gaps and limitations](#), these models do not recapitulate the complexity of cells, structures, and especially the biomolecular composition of the skin. In fact, the human skin comprises a broad range of molecules such as fibrous proteins, proteoglycans, glycoproteins, soluble growth

factors, and signaling molecules all of which are precisely arranged within each skin layer and skin appendages promoting proliferation, migration, and differentiation of cells (Watt and Fujiwara 2011; Holzl et al. 2016). The main components of the dermal matrix are the fibril-forming proteins: collagen types I, III, and V, which represent, respectively, 90%, 10%, and 2% of the total collagen content of the dermis (Smith et al. 1986; Watt and Fujiwara 2011). Besides collagens, the glycoproteins such as elastin and fibronectin, and the proteoglycans dermatan sulfate, hyaluronic acid, and perlecan are also distributed within the dermal compartment (Krieg and Aumailley 2011; Watt and Fujiwara 2011). At the dermal–epidermal junction, type IV collagen, the glycoproteins laminin and fibronectin, and several other proteoglycans form a thin bilayer membrane known as the basement membrane (Yurchenco and Schittny 1990; Krieg and Aumailley 2011). This network of filaments regulates the proliferation, differentiation, and adhesion of epidermal cells to the dermal compartment (Yurchenco and Schittny 1990). The skin appendages also contain a specific composition and precise distribution of biomolecules. Glycosaminoglycans and proteoglycans such as versican, perlecan, dermatan sulfate, decorin, and sydecans are precisely arranged in the different compartment of the hair follicle (for further details please see reviews by Bernard (2016) and Smith and Melrose (2015)). These glycans are indirectly associated with the regulation of hair follicle growth cycle through the activation and modulation of signaling molecules such as Wnt and growth factors such as TGF- β and EGF, as well as mediation of molecule–receptor interactions (also reviewed in further details by Bernard [2016]).

8.4.3 Incorporation of growth factors in bioinks

Soluble signaling molecules such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), fibroblast growth factor (FGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) also play an important role in maintaining proper skin homeostasis (Yildirim et al. 2012). *In vitro*, these molecules, typically supplemented in media, have been shown to regulate proliferation, migration, and differentiation of skin cells (Hasskarl et al. 2006; Peplow and Chatterjee 2013; Fuchs 1990). The inclusion of these growth factors in scaffolds has also been investigated. For example, the immobilization of EGF in gelatin sheets or biodegradable microspheres has shown improved wound healing in animal models (Ulubayram et al. 2001; Tanaka et al. 2005). In another study, a 3D-bioprinted matrix containing EGF increased proliferation of sweat gland cells (Huang et al. 2010). These biomolecules and cells, combined in a relevant manner, can be used to fabricate the bioinks for printing different layers and structures of the skin, generating a skin model with increased complexity and functionality. A comprehensive effort to develop bioinks that could sufficiently mimic the complexity of human skin is lacking. As a result, 3D-bioprinting approaches typically mimic the standard protocols for *manual* skin reconstruction employing relatively simple combinations of biomaterials.

In the first proof-of-concept work on 3D bioprinting of skin, Lee et al. (2014) demonstrated the feasibility of printing human primary cells along with proteins to form a tissue-like structure using an inkjet-based printer. In this work, the epidermal bioink (keratinocytes suspended in cell culture media) was printed on top of a dermal compartment constructed by consecutively printing layers of one bioink composed of rat tail type-I collagen, a second bioink composed of fibroblasts in medium/collagen, and a third bioink composed of keratinocytes in medium (Lee et al. 2014). After incubation for 14 days at the air–liquid interface, the 3D-bioprinted structures presented a dermal compartment and an incompletely stratified epidermis (Lee et al. 2014). Also employing an inkjet-based 3D-bioprinting technology, Rimann et al. (2016) printed successive layers of human fibroblasts and in a commercial ready-to-use polyethylene glycol (PEG)-based bioink followed by deposition of keratinocytes. They have demonstrated that the dermal compartment formed by alternating layers of the photocross-linked bioink and fibroblasts supported production of ECM and differentiation of keratinocytes (Rimann et al. 2016). In a different approach, Koch et al. (2012) employed a laser-assisted 3D bioprinter to deposit bioinks comprising fibroblasts and keratinocytes suspended in type I collagen. In this platform, the bioinks were first manually scattered onto the absorbent surface of a specific glass slide rather than being held in *biocartridges*. After laser incidence on the opposite side of this absorbent surface, the bioinks were released from the glass and precisely deposited onto the surface below or on top of the previously printed layers. In their model, the dermal bioink printed on top of a stabilizing sheet of Matriderm™, followed by deposition of epidermal bioink also resulted in a skin model comprising an incompletely stratified epidermis supported by the dermal layers (Koch et al. 2012). Recently, Cubo et al. (2016) successfully printed skin using a plasma-derived fibrin dermal bioink containing fibroblasts and an epidermal bioink comprising the keratinocytes. To overcome the issue of the fast coagulation rate of fibrin when in contact with calcium chloride, the *dermal bioink* was generated via an inline mixing of three different bioinks: (1) fibroblasts suspended in media, (2) tranexamic acid and human plasma containing fibrinogen, and (3) calcium chloride solution (Cubo et al. 2016). Using this protocol, the group printed a large skin sample (100 cm²) in less than 35 min, and the resulting tissue was putatively similar to native human skin (Cubo et al. 2016).

The examples cited earlier demonstrate that 3D-bioprinting technology can be used to deposit bioinks formulated from individual matrix components and cells to recreate the epidermal and dermal compartments. Nonetheless, the resulting structures achieved in these works fall short of reaching the complexity and functionality of native human skin. The design of optimized bioinks can potentiate the creation of *in vitro* skin models with functionality comparable to native human skin in combination with the 3D-bioprinting technology. Furthermore, similar design strategies could be employed in the development of bioinks for 3D bioprinting of other tissues and organs.

8.5 Perspectives and future directions

A rich history of discoveries and innovations in the field of TE over the past four decades has set the stage for new technologies such as 3D printing to further advance the goal of fabricating tissue and organ systems in the laboratory for applications in efficacy testing and regenerative medicine. The ability to engineer biological structures precisely and rapidly at supracellular, cellular, and subcellular length-scales has opened many new opportunities to approach the complexity and sophistication of native biological systems. We are already witnessing a clinical translation and adoption of several early examples, and the future will continue to bring many more. Nonetheless, many challenges remain that need to be addressed in the regulatory processes. Given that skin has been the first engineered tissue to gain clinical acceptance, it is likely that bioprinted skin tissues will also follow suit.

References

- Abaci, H. E., Z. Guo, A. Coffman, B. Gillette, W. H. Lee, S. K. Sia, and A. M. Christiano. 2016. Human skin constructs with spatially controlled vasculature using primary and iPSC-derived endothelial cells. *Advanced Healthcare Materials* 5 (14): 1800–1807. doi:10.1002/adhm.201500936.
- Balana, M. E., H. E. Charreau, and G. J. Leiros. 2015. Epidermal stem cells and skin tissue engineering in hair follicle regeneration. *World Journal of Stem Cells* 7 (4): 711–727. doi:10.4252/wjsc.v7.i4.711.
- Beaumont, K. A., N. Mohana-Kumaran, and N. K. Haass. 2013. Modeling melanoma in vitro and in vivo. *Healthcare* 2 (1): 27–46. doi:10.3390/healthcare2010027.
- Bechetoille, N., C. Dezutter-Dambuyant, O. Damour, V. Andre, I. Orly, and E. Perrier. 2007. Effects of solar ultraviolet radiation on engineered human skin equivalent containing both Langerhans cells and dermal dendritic cells. *Tissue Engineering* 13 (11): 2667–2679. doi:10.1089/ten.2006.0405.
- Bernard, B. A. 2016. Advances in understanding hair growth. *F1000Research* 5. doi:10.12688/f1000research.7520.1.
- Bernard, B. 2017. The hair follicle enigma. *Experimental Dermatology* 1–6. doi:10.1111/exd.13337.
- Bert, P. 1865. Notes sur un cas de greffe animale. *Comptes Rendus de La Societe de Biologie* 61: 587.
- Bibb, R., N. Nottrodt, and A. Gillner. 2016. Artificial vascularized scaffolds for 3D-tissue regeneration—a report of the ArtiVasc 3D project. *International Journal of Bioprinting* 2 (1): 93–102. doi:10.18063/IJB.2016.01.004.
- Blais, M., M. Grenier, and F. Berthod. 2009. Improvement of nerve regeneration in tissue-engineered skin enriched with schwann cells. *The Journal of Investigative Dermatology* 129 (12): 2895–2900. doi:10.1038/jid.2009.159.
- Boehnke, K., N. Mirancea, A. Pavesio, N. E. Fusenig, P. Boukamp, and H. J. Stark. 2007. Effects of fibroblasts and microenvironment on epidermal regeneration and tissue function in long-term skin equivalents. *European Journal of Cell Biology* 86 (11–12): 731–746. doi:10.1016/j.ejcb.2006.12.005.
- Botham, P. A., L. K. Earl, J. H. Fentem, R. Roguet, and J. J. M. Van De Sandt. 1998. Alternative methods for skin irritation testing: The current status. *ATLA Alternatives to Laboratory Animals* 26 (2): 195–211.

- Braverman, I. M. 1989. Ultrastructure and organization of the cutaneous microvasculature in normal and pathologic states. *Journal of Investigative Dermatology* 93 (s2): 2S–9S. doi:10.1111/1523-1747.ep12580893.
- Braverman, I. M. 1997. The cutaneous microcirculation: Ultrastructure and microanatomical organization. *Microcirculation* 4 (3): 329–340. doi:10.3109/10739689709146797.
- Braverman, I. M. 2000. The cutaneous microcirculation. *Journal of Investigative Dermatology Symposium Proceedings* 5 (1): 3–9. doi:10.1046/j.1087-0024.2000.00010.x.
- Braverman, I. M., and A. Keh-Yen. 1981. Ultrastructure of the human dermal microcirculation. III. The vessels in the mid- and lower dermis and subcutaneous fat. *The Journal of Investigative Dermatology* 77 (3): 297–304. doi:10.1111/1523-1747.ep12482470.
- Braverman, I. M., and J. Sibley. 1982. Role of the microcirculation in the treatment and pathogenesis of psoriasis. *The Journal of Investigative Dermatology* 78 (1): 12–17. doi:10.1111/1523-1747.ep12497850.
- Braverman, I. M., and J. Sibley. 1990. Ultrastructural and three-dimensional analysis of the contractile cells of the cutaneous microvasculature. *The Journal of Investigative Dermatology* 95 (1): 90–96. doi:10.1111/1523-1747.ep12874034.
- Braverman, I. M., and A. Yen. 1977. Ultrastructure of the human dermal microcirculation. *Journal of Investigative Dermatology* 68 (1): 44–52. doi:10.1111/1523-1747.ep12485165.
- Brenner, M., and V. J. Hearing. 2008. The protective role of melanin against UV damage in human skin. *Photochemistry and Photobiology* 84 (3): 539–549. doi:10.1111/j.1751-1097.2007.00226.x.
- Brohem, C. A., L. B. da Silva Cardeal, M. Tiago, M. S. Maria, S. Soengas, S. B. de Moraes Barros, and S. S. Maria-Engler. 2011. Artificial skin in perspective: Concepts and applications. *Pigment Cell & Melanoma Research* 24 (1): 35–50. doi:10.1111/j.1755-148X.2010.00786.x.
- Cai, Y., C. Fleming, and J. Yan. 2012. New insights of T cells in the pathogenesis of psoriasis. *Cellular and Molecular Immunology* 9 (4): 302–309. doi:10.1038/cmi.2012.15.
- Capla, J. M., D. J. Ceradini, O. M. Tepper, M. J. Callaghan, K. A. Bhatt, R. D. Galiano, J. P. Levine, and G. C. Gurtner. 2006. Skin graft vascularization involves precisely regulated regression and replacement of endothelial cells through both angiogenesis and vasculogenesis. *Plastic and Reconstructive Surgery* 117 (3): 836–844. doi:10.1097/01.prs.0000201459.91559.7f.
- Chau, D. Y. S., C. Johnson, S. Macneil, J. W. Haycock, and A. M. Ghaemmaghami. 2013. The development of a 3D immunocompetent model of human skin. *Biofabrication* 5 (3): 35011. doi:10.1088/1758-5082/5/3/035011.
- Chimene, D., K. K. Lennox, R. R. Kaunas, and A. K. Gaharwar. 2016. Advanced bioinks for 3D printing: A materials science perspective. *Annals of Biomedical Engineering* 44 (6): 2090–2102. doi:10.1007/s10439-016-1638-y.
- Chouhan, K., A. Kumar, and A. J. Kanwar. 2013. Body hair transplantation in vitiligo. *Journal of Cutaneous and Aesthetic Surgery* 6 (2): 111–112. doi:10.4103/0974-2077.112675.
- Chunmeng, S., and C. Tianmin. 2004. Skin: A promising reservoir for adult stem cell populations. *Medical Hypotheses* 62 (5): 683–688. doi:10.1016/j.mehy.2003.12.022.
- Converse, J. M., J. Smahel, D. L. Ballantyne, and A. D. Harper. 1975. Inosculation of vessels of skin graft and host bed: A fortuitous encounter. *British Journal of Plastic Surgery* 28 (1955): 274–282.
- Cubo, N., M. Garcia, J. F. Del Canizo, D. Velasco, and J. L. Jorcano. 2016. 3D bioprinting of functional human skin: Production and in vivo analysis. *Biofabrication* 9 (1): 15006. doi:10.1088/1758-5090/9/1/015006.
- Dezutter-Dambuyant, C., A. Black, N. Bechetoille, C. Bouez, S. Marechal, C. Auxenfans, V. Cenizo, P. Pascal, E. Perrier, and O. Damour. 2006. Evolutionary skin reconstructions: From the dermal collagen-glycosaminoglycan-chitosane substrate to an immunocompetent reconstructed skin. *Bio-Medical Materials and Engineering* 16 (4 Suppl): S85–S94.

- Eckert, R. L., and E. A. Rorke. 1989. Molecular biology of keratinocyte differentiation. *Environmental Health Perspectives* 80: 109.
- Fitzpatrick, T. B. 1988. The validity and practicality of sun-reactive skin types I through VI. *Archives of Dermatology* 124 (6): 869–871.
- Frueh, F. S., M. D. Menger, N. Lindenblatt, P. Giovanoli, and M. W. Laschke. 2016. Current and emerging vascularization strategies in skin tissue engineering. *Critical Reviews in Biotechnology* 8551: 1–13. doi:10.1080/07388551.2016.1209157.
- Fuchs, E. 1990. Epidermal differentiation: The bare essentials. *Journal of Cell Biology* 111 (6): 2807–2814. doi:10.1083/jcb.111.6.2807.
- Garrè, C. 1889. Ueber die histologischen Vorgänge bei der Anheilung der Thiersch'schen Transplantationen. *Brun's Beitr Klin Chir* 4: 625–652.
- Gibot, L., T. Galbraith, J. Huot, and F. A. Auger. 2010. A preexisting microvascular network benefits in vivo revascularization of a microvascularized tissue-engineered skin substitute. *Tissue Engineering Part A* 16 (10): 3199–3206. doi:10.1089/ten.tea.2010.0189.
- Gibot, L., T. Galbraith, J. Huot, and F. A. Auger. 2013. Development of a tridimensional microvascularized human skin substitute to study melanoma biology. *Clinical and Experimental Metastasis* 30 (1): 83–90. doi:10.1007/s10585-012-9511-3.
- Griffiths, M., N. Ojeh, R. Livingstone, R. Price, and H. Navsaria. 2004. Survival of apligraf in acute human wounds. *Tissue Engineering* 10 (7): 1180–1195. doi:10.1089/ten.2004.10.1180.
- Harper, R. A., and G. Grove. 1979. Human skin fibroblasts derived from papillary and reticular dermis: Differences in growth potential in vitro. *Science* 204 (4392): 526–527.
- Hasskarl, J., P. Velupillai, and K. Munger. 2006. Increased in vitro lifespan of primary human keratinocytes correlates with decreased migration. *Journal of Investigative Dermatology* 126 (5): 1179–1181. doi:10.1038/sj.jid.5700205.
- Higgins, C. A., J. C. Chen, J. E. Cerise, C. A. B. Jahoda, and A. M. Christiano. 2013. Microenvironmental reprogramming by three-dimensional culture enables dermal papilla cells to induce de novo human hair-follicle growth. *Proceedings of the National Academy of Sciences of the United States of America* 110 (49): 19679–19688. doi:10.1073/pnas.1309970110.
- Higgins, C. A., and A. M. Christiano. 2014. Regenerative medicine and hair loss: How hair follicle culture has advanced our understanding of treatment options for androgenetic alopecia. *Regenerative Medicine* 9 (1): 101–111. doi:10.2217/rme.13.87.
- Hoch, E., G. E. M. Tovar, and K. Borchers. 2014. Bioprinting of artificial blood vessels: Current approaches towards a demanding goal. *European Journal of Cardio-Thoracic Surgery* 46 (5): 767–778. doi:10.1093/ejcts/ezu242.
- Holzl, K., S. Lin, L. Tytgat, S. Van Vlierberghe, L. Gu, and A. Ovsianikov. 2016. Bioink properties before, during and after 3D bioprinting. *Biofabrication* 8 (3): 32002. doi:10.1088/1758-5090/8/3/032002.
- Huang, S., Y. Xu, C. Wu, D. Sha, and X. Fu. 2010. In vitro constitution and in vivo implantation of engineered skin constructs with sweat glands. *Biomaterials* 31 (21): 5520–5525. doi:10.1016/j.biomaterials.2010.03.060.
- Huang, S., B. Yao, J. Xie, and X. Fu. 2016. 3D bioprinted extracellular matrix mimics facilitate directed differentiation of epithelial progenitors for sweat gland regeneration. *Acta Biomaterialia* 32 170–177. doi:10.1016/j.actbio.2015.12.039.
- Ito, M., Y. Liu, Z. Yang, J. Nguyen, F. Liang, R. J. Morris, and G. Cotsarelis. 2005. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nature Medicine* 11 (12): 1351–1354. doi:10.1038/nm1328.
- Jung, E. C., and H. I. Maibach. 2015. Animal models for percutaneous absorption. *Journal of Applied Toxicology* 35 (1): 1–10. doi:10.1002/jat.3004.

- Kang, B. M., M. H. Kwack, M. K. Kim, J. C. Kim, and Y. K. Sung. 2012. Sphere formation increases the ability of cultured human dermal papilla cells to induce hair follicles from mouse epidermal cells in a reconstitution assay. *The Journal of Investigative Dermatology* 132 (1): 237–239. doi:10.1038/jid.2011.250.
- Kirsner, R. S. S., V. Falanga, F. A. Kerdell, M. H. Katz, and W. H. Eaglstein. 1996. Skin grafts as pharmacological agents: Pre-wounding of the donor site. *The British Journal of Dermatology* 135 (2): 292–296. doi:10.1111/j.1365-2133.1996.tb01164.x.
- Klemp, P. 1987. Cutaneous and subcutaneous blood flow measurements in psoriasis. *Danish Medical Bulletin* 34 (3): 147–159.
- Knowlton, S., B. Yenilmez, and S. Tasoglu. 2016. Towards single-step biofabrication of organs on a chip via 3D printing. *Trends in Biotechnology* 34 (9): 685–688. doi:10.1016/j.tibtech.2016.06.005.
- Koch, L., A. Deiwick, S. Schlie, S. Michael, M. Gruene, V. Coger, D. Zychlinski et al. 2012. Skin tissue generation by laser cell printing. *Biotechnology and Bioengineering* 109 (7): 1855–1863. doi:10.1002/bit.24455.
- Krieg, T., and M. Aumailley. 2011. The extracellular matrix of the dermis: Flexible structures with dynamic functions. *Experimental Dermatology* 20 (8): 689–695. doi:10.1111/j.1600-0625.2011.01313.x.
- Krueger, G. G., and C. M. Jorgensen. 1990. Experimental models for psoriasis. *Journal of Investigative Dermatology* 95 (5): S56–S58. doi:10.1111/1523-1747.ep12505791.
- Lazic, T., and V. Falanga. 2011. Bioengineered skin constructs and their use in wound healing. *Plastic and Reconstructive Surgery* 127 Suppl: 75S–90S. doi:10.1097/PRS.0b013e3182009d9f.
- Lee, M. L., T. To, E. Nicholson, and L. Schrieber. 1994. Endothelial cell adhesion molecules in psoriasis. *The Australasian Journal of Dermatology* 35 (2): 65–70.
- Lee, V., G. Singh, J. P. Trasatti, C. Bjornsson, X. W. Xu, T. N. Tran, S. S. Yoo, G. H. Dai, and P. Karande. 2014. Design and fabrication of human skin by three-dimensional bioprinting. *Tissue Engineering Part C-Methods* 20 (6): 473–484. doi:10.1089/ten.tec.2013.0335.
- Lim, C. P., T. T. Phan, I. J. Lim, and X. Cao. 2009. Cytokine profiling and stat3 phosphorylation in epithelial-mesenchymal interactions between keloid keratinocytes and fibroblasts. *The Journal of Investigative Dermatology* 129 (4): 851–861. doi:10.1038/jid.2008.337.
- Lin, B., Y. Miao, J. Wang, Z. Fan, L. Du, Y. Su, B. Liu, Z. Hu, and M. Xing. 2016. Surface tension guided hanging-drop: Producing controllable 3D spheroid of high-passaged human dermal papilla cells and forming inductive microtissues for hair-follicle regeneration. *ACS Applied Materials {&} Interfaces* 8 (9): 5906–5916. doi:10.1021/acsami.6b00202.
- Liu, N., S. Huang, B. Yao, J. Xie, X. Wu, and X. Fu. 2016. 3D bioprinting matrices with controlled pore structure and release function guide in vitro self-organization of sweat gland. *Scientific Reports* 6: 34410.
- Lu, C., and E. Fuchs. 2014. Sweat gland progenitors in development, homeostasis, and wound repair. *Cold Spring Harbor Perspectives in Medicine* 4 (2): a015222. doi:10.1101/cshperspect.a015222.
- Maas-Szabowski, N., A. Stärker, and N. E. Fusenig. 2003. Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-Alpha. *Journal of Cell Science* 116 (Pt 14): 2937–2948. doi:10.1242/jcs.00474.
- Mahjour, S. B., F. Ghaffarpasand, and H. Wang. 2012. Hair follicle regeneration in skin grafts: Current concepts and future perspectives. *Tissue Engineering. Part B, Reviews* 18 (1): 15–23. doi:10.1089/ten.teb.2011.0064.
- Malda, J., and J. Groll. 2016. A step towards clinical translation of biofabrication. *Trends in Biotechnology* 34 (5): 356–357. doi:10.1016/j.tibtech.2016.03.003.

- Mathes, S. H., H. Ruffner, and U. Graf-Hausner. 2014. The use of skin models in drug development. *Advanced Drug Delivery Reviews* 69–70: 81–102. doi:10.1016/j.addr.2013.12.006.
- Meier, F., M. Nesbit, M. Y. Y. Hsu, B. Martin, P. Van Belle, D. E. Elder, G. Schaumburg-Lever et al. 2000. Human melanoma progression in skin reconstructs: Biological significance of bFGF. *The American Journal of Pathology* 156 (1): 193–200. doi:10.1016/S0002-9440(10)64719-0.
- Metcalf, A. D., and M. W. J. Ferguson. 2007. Tissue engineering of replacement skin: The crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *Journal of the Royal Society Interface* 4 (14): 413–437. doi:10.1098/rsif.2006.0179.
- Miao, Y., Y. B. Sun, B. C. Liu, J. D. Jiang, and Z. Q. Hu. 2014. Controllable production of transplantable adult human high-passage dermal papilla spheroids using 3D matrigel culture. *Tissue Engineering. Part A* 20 (17–18): 2329–2338. doi:10.1089/ten. TEA.2013.0547.
- Miller, A. J., and M. C. Jr Mihm. 2006. Melanoma. *The New England Journal of Medicine* 355 (1): 51–65. doi:10.1056/NEJMra052166.
- Moulin, V. J., D. Mayrand, A. Laforce-Lavoie, S. Larochelle, and H. Genest. 2011. In vitro culture methods of skin cells for optimal skin reconstruction by tissue engineering. In *Regenerative Medicine and Tissue Engineering - Cells and Biomaterials* D. Eberli (Ed). InTech. doi:10.5772/20341.
- Muñoz-Abraham, A. S., M. I. Rodriguez-Davalos, A. Bertacco, B. Wengerter, J. P. Geibel, and D. C. Mulligan. 2016. 3D printing of organs for transplantation: Where are we and where are we heading? *Current Transplantation Reports* 3 (1): 93–99. doi:10.1007/s40472-016-0089-6.
- Murphy, S. V., and A. Atala. 2014. 3D bioprinting of tissues and organs. *Nature Biotechnology* 32 (8): 773–785. doi:10.1038/nbt.2958.
- Nakao, K., R. Morita, Y. Saji, K. Ishida, Y. Tomita, M. Ogawa, M. Saitoh, Y. Tomooka, and T. Tsuji. 2007. The development of a bioengineered organ germ method. *Nature Methods* 4 (3): 227–230. doi:10.1038/nmeth1012.
- Netzlaff, F., C.-M. Lehr, P. W. Wertz, and U. F. Schaefer. 2005. The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *European Journal of Pharmaceutics and Biopharmaceutics* 60 (2): 167–178. doi:10.1016/j.ejpb.2005.03.004.
- Ng, W. L., S. Wang, W. Y. Yeong, and M. W. Naing. 2016. Skin bioprinting: Impending reality or fantasy? *Trends in Biotechnology* 34 (9): 689–699. doi:10.1016/j.tibtech.2016.04.006.
- Niemann, C., and V. Horsley. 2012. Development and homeostasis of the sebaceous gland. *Seminars in Cell & Developmental Biology* 23 (8): 928–936. doi:10.1016/j.semdb.2012.08.010.
- Ohshima, M., and O. Veraitch. 2013. Strategies to enhance epithelial–mesenchymal interactions for human hair follicle bioengineering. *Journal of Dermatological Science* 70 (2): 78–87. doi:10.1016/j.jdermsci.2013.02.004.
- Ouyang, L., R. Yao, Y. Zhao, and W. Sun. 2016. Effect of bioink properties on printability and cell viability for 3D bioplotting of embryonic stem cells. *Biofabrication* 8 (3): 35020. doi:10.1088/1758-5090/8/3/035020.
- Pan, J., S. Y. Chan, J. E. A. Common, S. Amini, A. Miserez, E. Birgitte Lane, and L. Kang. 2013. Fabrication of a 3D hair follicle-like hydrogel by soft lithography. *Journal of Biomedical Materials Research. Part A* 101 (11): 3159–3169. doi:10.1002/jbm.a.34628.
- Panteleyev, A. A., C. A. Jahoda, and A. M. Christiano. 2001. Hair follicle predetermination. *Journal of Cell Science* 114 (Pt 19): 3419–3431.
- Pasparakis, M., I. Haase, and F. O. Nestle. 2014. Mechanisms regulating skin immunity and inflammation. *Nature Reviews Immunology* 14 (5): 289–301. doi:10.1038/nri3646.

- Peplow, P. V., and M. P. Chatterjee. 2013. A review of the influence of growth factors and cytokines in in vitro human keratinocyte migration. *Cytokine* 62 (1): 1–21. doi:10.1016/j.cyt.2013.02.015.
- Perkins, M. A., R. Osborne, F. R. Rana, A. Ghassemi, and M. K. Robinson. 1999. Comparison of in vitro and in vivo human skin responses to consumer products and ingredients with a range of irritancy potential. *Toxicological Sciences* 48 (2): 218–229. doi:10.1093/toxsci/48.2.218.
- Phillips, T. J., J. Manzoor, A. Rojas, C. Isaacs, P. Carson, M. Sabolinski, J. Young, and V. Falanga. 2002. The longevity of a bilayered skin substitute after application to venous ulcers. *Archives of Dermatology* 138 (8): 1079–1081. doi:10.1001/archderm.138.8.1079.
- Regnier, M., A. Patwardhan, A. Scheynius, and R. Schmidt. 1998. Reconstructed human epidermis composed of keratinocytes, melanocytes and langerhans cells. *Medical and Biological Engineering and Computing* 36 (6): 821–824.
- Reijnders, C. M. A., A. van Lier, S. Roffel, D. Kramer, R. J. Scheper, and S. Gibbs. 2015. Development of a full-thickness human skin equivalent in vitro model derived from TERT-immortalized keratinocytes and fibroblasts. *Tissue Engineering. Part A* 21 (17–18): 2448–2459. doi:10.1089/ten. TEA.2015.0139.
- Rimann, M., E. Bono, H. Annaheim, M. Bleisch, and U. Graf-Hausner. 2016. Standardized 3D bioprinting of soft tissue models with human primary cells. *Journal of Laboratory Automation* 21 (4): 496–509. doi:10.1177/2211068214567146.
- Schmidt-Ullrich, R., and R. Paus. 2005. Molecular principles of hair follicle induction and morphogenesis. *BioEssays* 27 (3): 247–261. doi:10.1002/bies.20184.
- Smith, L. T., K. A. Holbrook, and J. A. Madri. 1986. Collagen types I, III, and V in human embryonic and fetal skin. *The American Journal of Anatomy* 175 (4): 507–521. doi:10.1002/aja.1001750409.
- Smith, M. M., and J. Melrose. 2015. Proteoglycans in normal and healing skin. *Advances in Wound Care* 4 (3): 152–173. doi:10.1089/wound.2013.0464.
- Takagi, R., J. Ishimaru, A. Sugawara, K. Toyoshima, K. Ishida, M. Ogawa, K. Sakakibara et al. 2016. Bioengineering a 3D integumentary organ system from iPS cells using an in vivo transplantation model. *Science Advances* 2 (4): e1500887. doi:10.1126/sciadv.1500887.
- Tanaka, A., T. Nagate, and H. Matsuda. 2005. Acceleration of wound healing by gelatin film dressings with epidermal growth factor. *The Journal of Veterinary Medical Science* 67 (9): 909–913.
- Thangapazham, R. L., P. Klover, S. Li, J. A. Wang, L. Sperling, and T. N. Darling. 2014. A model system to analyse the ability of human keratinocytes to form hair follicles. *Experimental Dermatology*. doi:10.1111/exd.12424.
- Thiersch, C. 1869. Ueber sie entstehungsweise und operative behandlung der epispadie. *Arch Heilkunde* 10: 20.
- Toyoshima, K., K. Asakawa, N. Ishibashi, H. Toki, M. Ogawa, T. Hasegawa, T. Irié et al. 2012. Fully functional hair follicle regeneration through the rearrangement of stem cells and their niches. *Nature Communications* 3: 784.
- Tremblay, P. L., V. Hudon, F. Berthod, L. Germain, and F. Auger. 2005. Inosculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice. *American Journal of Transplantation* 5 (5): 1002–1010. doi:10.1111/j.1600-6143.2005.00790.x.
- Tsur, H., A. Daniller, and B. Strauch. 1980. Neovascularization of skin flaps: Route and timing. *Plastic and Reconstructive Surgery* 66 (1): 85–90.
- Uchi, H., H. Terao, T. Koga, and M. Furue. 2000. Cytokines and chemokines in the epidermis. *Journal of Dermatological Science* 24 (Suppl. 1): 29–38. doi:10.1016/S0923-1811(00)00138-9.

- Ulubayram, K., A. Nur Cakar, P. Korkusuz, C. Ertan, and N. Hasirci. 2001. EGF containing gelatin-based wound dressings. *Biomaterials* 22 (11): 1345–1356. doi:10.1016/S0142-9612(00)00287-8.
- van den Bogaard, E. H., D. Rodijk-Olthuis, P. A. M. Jansen, I. M. J. van Vlijmen-Willems, P. E. van Erp, I. Joosten, P. L. J. M. Zeeuwen, and J. Schalkwijk. 2012. Rho kinase inhibitor Y-27632 prolongs the life span of adult human keratinocytes, enhances skin equivalent development, and facilitates lentiviral transduction. *Tissue Engineering. Part A* 18 (17–18): 1827–1836. doi:10.1089/ten.tea.2011.0616.
- Velasquillo, C., E. A. Galue, L. Rodriguez, C. Ibarra, and L. G. Ibarra-Ibarra. 2013. Skin 3D bioprinting. Applications in cosmetology. *Journal of Cosmetics, Dermatological Sciences and Applications* 3 (1A): 85–89. doi:10.4236/jcdsa.2013.31A012.
- Vermeulen, N., G. Haddow, T. Seymour, A. Faulkner-Jones, and W. Shu. 2017. 3D bioprint me: A socioethical view of bioprinting human organs and tissues. *Journal of Medical Ethics* 1–7. doi:10.1136/medethics-2015-103347.
- Vijayavenkataraman, S, W. F. Lu, and J. Y. H. Fuh. 2016. 3D bioprinting of skin: A state-of-the-art review on modelling, materials, and processes. *Biofabrication* 8 (3): 32001. doi:10.1088/1758-5090/8/3/032001.
- Watt, F. M., and H. Fujiwara. 2011. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harbor Perspectives in Biology* 3 (4): a005124. doi:10.1101/cshperspect.a005124.
- Yano, K., L. F. Brown, and M. Detmar. 2001. Control of hair growth and follicle size by VEGF-mediated angiogenesis. *Journal of Clinical Investigation* 107 (4): 409–417.
- Yen, A., and I. M. Braverman. 1976. Ultrastructure of the human dermal microcirculation: The horizontal plexus of the papillary dermis. *Journal of Investigative Dermatology* 66 (3): 131–142. doi:10.1111/1523-1747.ep12481678.
- Yildirim, L., N. T. K. Thanh, and A. M. Seifalian. 2012. Skin regeneration scaffolds: A multimodal bottom-up approach. *Trends in Biotechnology* 30 (12): 638–648. doi:10.1016/j.tibtech.2012.08.004.
- Yu, H. S. 2002. Melanocyte destruction and repigmentation in vitiligo: A model for nerve cell damage and regrowth. *Journal of Biomedical Science* 9 (6 Pt 2): 564–573.
- Yurchenco, P. D., and J. C. Schittny. 1990. Molecular architecture of basement membranes. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 4 (6): 1577–1590.
- Zhang, Z., and B. B. Michniak-Kohn. 2012. Tissue engineered human skin equivalents. *Pharmaceutics* 4 (1): 26–41. doi:10.3390/pharmaceutics4010026.

Chapter 9 Bioprinting vascular networks

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9.1 Introduction

Recent developments in three-dimensional (3D)-bioprinting technology enable the efficient fabrication of thick and complex tissues. Numerous 3D-bioprinting techniques have been developed to create sophisticated structures in a wide range of scales (from micrometer to centimeter) with various biomaterials (Datta et al. 2017; Ozbolat et al. 2017). Unlike traditional 3D printing that prints inorganic materials and needs minimal post-processing after the completion of printing, bioprinting involves more complicated components such as suitable cells and printable biomaterials and also requires substantial post-printing procedures. In order to develop tissue-specific functions, long-term culture with appropriate stimuli is necessary for most of the engineered tissues.

Vascularization of engineered tissues plays an important role in maintaining tissue viability during the long-term post-printing maturation (Kaully et al. 2009; Lovett et al. 2009; Phelps and Garcia 2010; Khademhosseini and Langer 2016). Vascular networks with perfusion provide an adequate supply of nutrient and oxygen, thus maintaining viability and preventing necrosis of thick tissues during maturation period. Vasculatures embedded within engineered tissues also provide backbone structures to recapitulate architectural features of the counterpart native tissue and enable easy integration into the host circulatory system after transplantation.

To address the vascularization issues, numerous bioprinting methodologies have been developed by (1) improving software and/or hardware platform;

(2) designing printing patterns and procedures; and (3) developing and fine-tuning new bioink materials. In this chapter, various bioprinting techniques for vascularized tissue fabrication will be reviewed in two categories. The first category includes bioprinting approaches that directly fabricate vascular wall structures. The second category of bioprinting techniques exploits an approach to first generate hollow channels within 3D matrix and subsequently endothelialize the channels.

9.2 Bioprinting vascular walls

The fabrication of tissue-engineered vascular graft (TEVG) is one of the most clinically-relevant applications of bioprinted vessels. For small-diameter vasculatures (diameter < 6 mm) that are commonly used for coronary artery bypass surgeries, the autograft vessel sources are very limited. The failure of vascular grafts is often caused by mismatched vessel properties (e.g., vessel diameter, compliance, damage to endothelial cells) and host-dependent side effects such as thrombosis, atherosclerosis, initial hyperplasia, and infection. TEVG has a great potential for supplying vessel grafts tailored with desired mechanical, chemical, and biological properties. (Pashneh-Tala et al. 2015). Bioprinting technology is capable of generating complex architecture with precision and controlling various types of biomaterials; thus it can serve as a powerful tool to create vessel graft with customized features.

Apart from its use as a vessel graft, thick-walled bioprinted vasculatures have value as an *in vitro* tissue study model. By using bioprinting approaches, numerous 3D vessel designs can be invented to test the influence of diverse factors such as vascular structures, cell types, flow patterns, vascular compliances, and branching angles. For example, a bifurcated y-shaped vessel, in which the dimension, mechanical properties, and/or bioink composition of one vascular branch are different from the other branch, can be easily fabricated using the existing bioprinting platforms. In addition, thick vascular wall allows a prolonged durability of bioprinted constructs and possibly provides an extra space for cell proliferation and tissue development.

Scaffold-free stand-alone structure can be achieved by the use of high-viscous bioink (that can retain aspect ratio for a prolonged duration) and fast polymerization process. The use of highly viscous materials is often inevitable to achieve the required mechanical properties for vascular grafts. For this reason, extrusion-based bioprinting has been the most popular technique to generate thick-wall vascular structures. Norotte et al. (2009) fabricated small diameter vessels using extrusion bioprinting. In the research, smooth muscle cells and fibroblasts were aggregated into tissue units, which serve as a cellular bioink. Agarose, used as

a molding template material, was printed alongside with the printed tissue unit filaments (Figure 9.1a). Agarose is nonadherent to cells, thus it can maintain the original printed architectures without a fusion with cellular rods. The printed tissue units were fused within 2–4 days of post-printing culture, thus creating small diameter vasculature (Figure 9.1A.a, A.b). Agarose rods were removed after the tissue integration, and then the vessel was transferred into bioreactors for further maturation process.

Similar to the agarose that aids to keep the original printed shape, supporting baths in emulsion form have been actively used for the fabrication of scaffold-free, stand-alone tubular structures. In general, extrusion nozzles are immersed in the supporting bath, the printed patterns are solidified, and then the supporting bath materials are removed. The supporting solution provides buoyant forces to support bioprinted structures against collapsing (Bhattacharjee et al. 2015; Christensen et al. 2015; Hinton et al. 2015; Highley et al. 2015). Hinton et al. (2015) printed a mixture of alginate, fibrin, and collagen into gelatin slurry supporting bath. The supporting solution contains cross-linking agents, calcium chloride, and thrombin for the rapid gelation of printed hydrogel mixture. At the completion of the printing process, gelatin was liquefied to release the printed vessel structure (Figure 9.1B.a, B.b). Bhattacharjee et al. (2015) used granular gel medium made from soft microparticles as a supporting bath. A hierarchically branched vascular structure was fabricated by using their printing platform (Figure 9.1C).

Although fabrication of tubular hydrogel structures can be achieved using most of the bioprinting modalities including extrusion-based (Norotte et al. 2009; Bhattacharjee et al. 2015; Hinton et al. 2015), light-based (Suri et al. 2011; Meyer et al. 2012; Hribar et al. 2015), and inkjet-based (Christensen et al. 2015; Tan et al. 2014) (Figure 9.1), the use of highly viscous bioinks in inkjet bioprinting may cause a nozzle clogging and a decreased printing resolution. The limitations can be compensated by printing supporting scaffold or mold along with tubular structure fabrication. Tan et al. (2014) first printed alginate mold that can hold tubular structures. Vascular spheroids containing smooth muscle cells and endothelial cells were then printed into the mold. A tubular vascular structure was generated by fusion of spheroids placed in the alginate mold after 3 days of culture. Christensen et al. (2015) also utilized an inkjet printing to enable horizontal and vertical bioprinting of vasculatures. They developed a predictive printing platform to compensate the structure deformations during printing process caused by the effect of gravity (Xu et al. 2012, 2014). Alginate bioink was projected into a calcium chloride solution, which serves as both a cross-linking agent and a support buoyant material. Through the horizontal and vertical printing of alginate gel, bifurcated and branched vessel-like structures were successfully fabricated (Christensen et al. 2015).

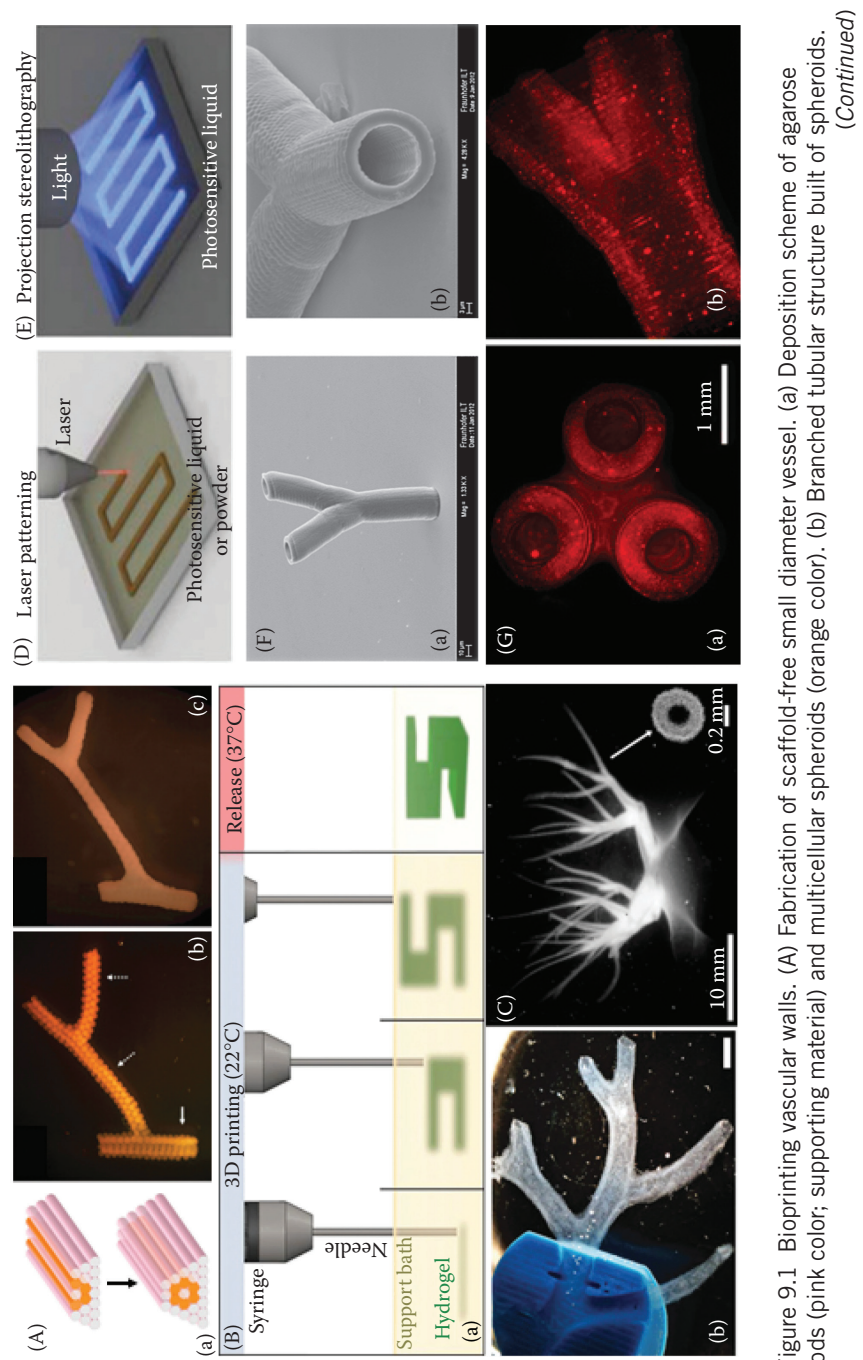


Figure 9.1 Bioprinting vascular walls. (A) Fabrication of scaffold-free small diameter vessel. (a) Deposition scheme of agarose rods (pink color; supporting material) and multicellular spheroids (orange color). (b) Branched tubular structure built of spheroids. (Continued)

Figure 9.1 (Continued) (c) Tissue maturation by the fusion of spheroids (Day 6). (Adapted from Norotte, C. et al., *Biomaterials*, 30, 5910–5917, 2009. With permission.) (B) Bioprinting using support bath. (a) Printing hydrogel precursor bioink into the thermoreversible support bath consisting of gelatin slurry and cross-linking agents. Gelatin bath was liquefied at 37°C after the completion of printing. (b) Arterial tree structure was printed in alginate. (Adapted from Hinton, T.J. et al., *Science Advances*, doi:10.1126/sciadv.1500758, 2015.) (C) Hierarchically branched tubular networks printed within granular gel bath. The granular gel was removed after the printing procedure. (Adapted from Bhattacharjee, T. et al., *Sci. Adv.*, doi:10.1126/sciadv.1500655, 2015. With permission.) (D and E) Schematics of light-based bioprinting. (D) Laser photopolymerization. (E) Projection-based photopolymerization. Repeat this step for every layer to create 3D structures. (Adapted from Albritton, J.L. and Miller, J.S., *Dis. Models Mech.*, doi:10.1242/dmm.025049, 2017.) (F) SEM images of branched tubular structure fabricated by two-photon polymerization. The structure is ~160 µm in height (a). The inner diameter and tube wall thickness is ~18 µm and ~3 µm (b). (Adapted from Meyer, W. et al., *J. Funct. Biomater.*, 3, 257–268, 2012. With permission.) (G) Branched tube created using projection-based bioprinting platform. Top view (a) and crosssection view (b). The structure was vitalized by embedded fluorescent particles. (Adapted from Suri, S. et al., *Biomed. Microdevices*, doi:10.1007/s10544-011-9568-9, 2011.)

Light-based bioprinting technology that employs photopolymerization (e.g., stereolithography) allows fast hydrogel polymerization and high resolution. It can also avoid the shear stress that may cause cellular damages during the extrusion- or inkjet-based printing procedure. However, the choice of bioink is limited to photocross-linking materials and laser irradiation may cause harmful effects on the encapsulated cells (Hribar et al. 2014; Zhu et al. 2016; Albritton and Miller 2017).

Laser patterning techniques make use of single- or multiphoton light sources to photopolymerize the monomer solution in serial printing manner (dot-by-dot polymerization) (Figure 9.1D) (Gebinoga et al. 2013; Albritton and Miller 2017). It allows high-resolution printing but requires long fabrication time for creating millimeter-scale structure. Meyer et al. (2012) created micrometer-scale vessel structures using multiphoton laser printing. They fabricated capillary scale of the branched structure, the inner diameter was 18 μm and the wall thickness was 4 μm (Figure 9.1F.a, F.b). Seeding cells inside the microtube or embedding cells within a wall of 4 μm thickness may not be feasible since the dimension of the structure is similar to or even smaller than single cell diameter. Also, a creation of complete capillary network using this technique will require tremendous time and resources. Nevertheless, this work successfully demonstrated the capability of bioprinting microstructure and can be combined with other tissue engineering approaches to recapitulate the architectural complexity of native tissues.

In the projection-based printing, a 2D pattern of light is projected on photocross-linking materials to create an entire plane of optical pattern at a single step (Figure 9.1E) (Gebinoga et al. 2013; Albritton and Miller 2017). This step is then repeated with different 2D patterns to construct a desired 3D structure. As the entire plane is polymerized at once, projection-based bioprinting is capable of high-speed printing with a great resolution and reproducibility. In addition, the interface effect between layers is minimized, courtesy of the fast and consistent printing time for each layer. Suri et al. (2011) created branched tubes using a projection-based printing method called digital micromirror device-based projection printing (DMD-PP) (Figure 9.1G.a, G.b). The construct was fabricated as a nerve conduit, but the same technique can be applied to create vessel-like tubular structures. The same research group also developed applications of DMD-PP for engineering microchannels (Huang et al. 2014) and vascular network patterns within hydrogel (Figure 9.2F) (Zhang et al. 2012).

Laser direct writing is a light-based bioprinting technologies, in which cells encapsulated within ribbon (laser-absorbing layer) are transferred to substrates by a laser pulse (Nahmias et al. 2005; Schiele et al. 2010). Xiong et al. (2015) developed a printing method for bifurcated tube construction using laser direct writing and a buoyant supporting bath. An alginate ribbon containing fibroblasts was prepared and projected into the calcium chloride bath by laser transfer. A bifurcated

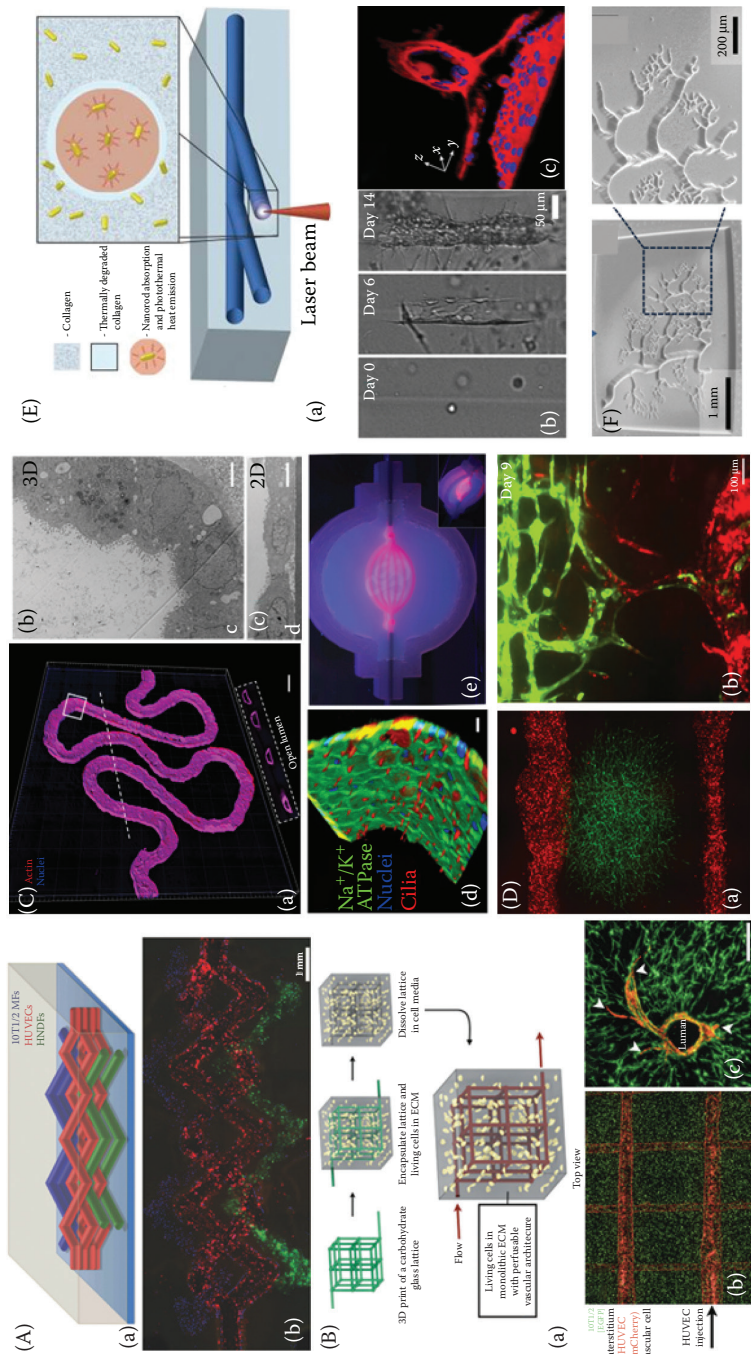


Figure 9.2 Bioprinting hollow channels followed by endothelialization. (A) Extrusion printing of zigzag patterns with three different cell types. (a) Schematics of printed construct. Blue=10T1/2 fibroblast within GelMA; Red=fugitive pluronic (sacrificial material); (Continued)

Figure 9.2 (Continued) Green=GFP HNF1B within GelMA; and Grey=plain GelMA. (b) Fluorescent image of the printed structure. Color coding is same as (b). Red filaments were evacuated to create open endothelialized channels. (Adapted from Kolesky, D.B. et al., *Adv. Mater.* 26, 3124–3130, 2014. With permission.) (B) 3D tissue structure containing fluidic channel lattice. (a) Schematics of the fabrication procedure: An open, interconnected, self-supporting carbohydrate glass lattice (sacrificial material) was printed by extrusion. ECM precursor containing cells filled the space around the lattice structure. After ECM solidification, the glass lattice was dissolved and endothelial cells were seeded to create fluidic vascular channels. (b) Endothelialized vascular channels (red) encapsulated within fibrin matrix containing fibroblasts (green) in the interstitial spaces. (c) Formation of single and multicellular sprouts from the edge of the fabricated vasculatures. (Adapted from Miller, J.S. et al., *Nat. Mater.*, 11, 768–774, 2012.) (C) 3D-convoluted renal proximal tubule. (a) Confocal image of proximal tubule with PTEC-coated open lumen. (b) PTEC grown within perfused 3D proximal tubule presented a polarized columnar epithelium with primary cilia formation, shown in TEM image (b, c) and confocal image (d). (e) Proximal tubule model (light purple color) constructed between two perfusable vascular networks (pink color), demonstrating how bioprinting can be combined with microfluidics. (Adapted from Homan, K.A. et al., *Sci. Rep.*, doi:10.1038/srep34845, 2016.) (D) Bioprinted multiscale vascular networks using inkjet printing. (a) Large (~1 mm) vascular channels (red) were fabricated by inkjet printing of gelatin (sacrificial material), then vasculogenesis process was induced to create capillary bed (green) connected to the large vessels. (b) Anastomosis between capillaries (green) and a large fluidic vascular channel (red). (Adapted from Lee et al., 2014a.) (E) 3D patterning of microchannels in collagen hydrogels with near-infrared femtosecond laser. (a) Schematics of microchannel fabrication process. A near-infrared laser light was focused on the desired 3D location to trigger photochemical degradation of collagen by stimulating embedded gold nanorods. (b) Embedded endothelial cells from the surrounding matrix migrated into the hollow channel. (c) The migrated cells formed an endothelium inside of the fabricated microchannel. (Adapted from Hribar, K.C. et al., *Sci. Rep.*, doi:10.1038/srep17203, 2015. With permission.) (F) Fabrication of biomimetic vascular structures by projection stereolithography. (Adapted from Zhang, A.P. et al., *Adv. Mater.*, doi:10.1002/adma.201202024, 2012. With permission.)

Y-shaped tube (~5 mm in diameter, >5 mm in height) structure was fabricated through layer-by-layer approach. Calcium chloride solution bath acted as a cross-linking solution and liquid supporter.

9.3 Bioprinting endothelialized hollow channels within thick matrix

One of the most popular methods to effectively generate vascular network within thick tissues is to first construct hollow channels and then to seed vascular cells on the inner surface of the channels (Figure 9.2B.a). The media perfusion through interconnected hollow channels can provide a sufficient supply of nutrient and oxygen even without vascular cell seeding (Miller et al. 2012). However, the incorporation of vascular cells provides numerous beneficial features for tissue growth and maturation. Endothelialized channels enable the engineered tissues (1) to maintain a long-term structural integrity of perfused channels; (2) to acquire functional features of endothelium such as barrier function, antithrombogenicity, and the capability to form new vessels; (3) to induce cell–cell interactions between vessel cells and adjacent tissue-specific cell types; (4) to generate flow-mediated stimuli within the tissue; and (5) to be more easily integrated into the host circulatory system after transplantation. These features often support various tissue-specific behaviors that can consequentially recapitulate morphological and functional features of the native tissues (Nerem and Seliktar 2001; Rouwkema et al. 2008).

Due to the nature of the fabrication approach, in which channels are created by removal of sacrificial materials from the bulk hydrogel, 3D-printed tissues that are fabricated through this method generally contain large void hydrogel space around vasculatures. The vessel structures mentioned in this section tend to consist of less viscous, transparent, or translucent hydrogel with reduced mechanical strengths. Due to the insufficient mechanical strength, these vessels may not be suitable for vessel grafts. However, the use of softer hydrogel and the empty perivascular areas makes it easy to embed other cell types or chemical components and furthermore to promote the motile behaviors of embedded cells (e.g., infiltration, migration, self-assembly, and proliferation). Thus, this approach presents a greater potential in engineering *in vitro*-vascularized tissue models for various tissue and organ types.

This approach that utilizes hollow-channel fabrication followed by endothelial cell seeding is compatible with the most of the bioprinting modalities (Figure 9.2 A–C: extrusion-based, D: inkjet, and E–F: laser-based printing). The hollow-channel network can be constructed by embedding sacrificial materials within 3D matrix (Miller et al. 2012; Bertassoni et al. 2014; Lee et al. 2014b; Kolesky et al. 2016)

or by the removal or ablation of hydrogel in channel shapes (Ilina et al. 2011; Sarig-Nadir et al. 2009; Hribar et al. 2015). Gelatin, pluronic, agarose, alginate, and carbohydrate are some examples of sacrificial materials that have been used to create hollow channels using bioprinting. The choice of fugitive method mainly depends on material properties. For gel–sol phase-changing materials, the fugitive inks are liquefied by temperature change (gelatin, pluronic) or by adding chelating reagents (alginate) and then evacuated through the fabricated channels (Lee 2014b; Jia et al. 2016; Kolesky et al. 2014, 2016). Nonadherent materials such as agarose can be removed by simple pulling-out method (Bertassoni et al. 2014). Carbohydrate glass dissolves in water, thus can be easily removed by adding buffer solution or culture medium (Miller et al. 2012).

Lee et al. (2014b) generated a perfusable functional vascular channel through ink-jet printing. Gelatin containing endothelial cells was printed at 37°C and then was quickly solidified at room temperature. The gelatin channel was enclosed within collagen hydrogel. Although gelatin was liquefied by heating, the encapsulated endothelial cells were attached to the inner surface of the channel, quickly forming endothelium. After then, the structure was connected to a perfusion system to wash out gelatin and start long-term flow culture (Lee et al. 2014b). The same group also developed a method to create multiscale vascular network with larger fluidic vessel and capillary bed. Large channels were fabricated by gelatin printing, and capillary bed was formed by natural vasculogenesis process in fibrin gel. The two different types of vessels were connected by angiogenic sprouting from the large channel and by following anastomosis with capillary vasculatures (Figure 9.2D.a, D.b) (Lee et al. 2014a).

Miller et al. (2012) used carbohydrate glass as a sacrificial material to create vascularized tissues. Carbohydrate glass was printed in 3D lattice structure in high temperature and then encased by hydrogel-containing cells. The self-supporting lattice structure was coated with a thin layer of poly(D-lactide-co-glycolide) (PDLGA) before the casting to prevent cell damage caused by dissolved carbohydrates. The sacrificial carbohydrate was dissolved by adding culture medium, and then endothelial cell seeding was performed by injecting cell suspension into the channels (Figure 9.2B.a). The seeded endothelial cells formed vascular lumen (Figure 9.2B.b) and eventually triggered vessel sprout formation in the presence of 10T1/2 cells in the interstitial region (Figure 9.2B.c). The interconnected channels supported the viability and metabolic function of densely populated hepatocytes within thick hydrogel.

Kolesky et al. (2014) demonstrated a fabrication of multilayer structure with three different cell types and perfusable vasculatures. The printing capability of their system was shown by creating multilayer of complicated (zigzag) patterns using two different thermoreversible hydrogel, pluronic, and GelMA (Figure 9.2A.a, A.b). In their other work, more complex vascularized tissue construct was engineered

through extrusion bioprinting. Self-supporting 3D lattice structures were created using two types of bioinks: (1) vascular fugitive ink (mixture of pluronic and thrombin) and (2) cell ink (mixture of cells, gelatin, fibrinogen, thrombin). The lattice structures were then encapsulated by hydrogel-containing gelatin, fibrinogen, cells, thrombin, and transglutaminase. Fibrinogen was rapidly polymerized by thrombin, and diffused transglutaminase from the interstitial hydrogel triggered slow cross-linking of gelatin and fibrin. The vascular ink was liquefied by cooling, and then endothelial cells were seeded throughout the hollow-channel network. They found that pre-processing of gelatin at high temperature (95°C) resulted in softer gels with lower viscosity, thereby allowing higher viability of embedded cells. The entire construct printed into perfusion chip (bioreactor) for long-term stable perfusion up to 30 days. The 3D-printing methods were applied to develop a dense osteogenic tissue by embedding human mesenchymal stem cells (hMSCs) and supplying osteogenic media for the long term. More mineral deposition, collagen type I deposition, and higher osteocalcin expression were observed near the perfused vasculatures. The results demonstrated the influence of vascularization in tissue maturation and functional development, showing a great tissue engineering application of bioprinted tissues (Kolesky et al. 2016).

Recently, same group developed 3D *in vitro* human kidney model by creating renal proximal tubules with convoluted shape. The engineered proximal tubules consist of an open lumen structure with a lining of proximal tubule epithelial cells (PTECs), embedded within hydrogel matrix (Figure 9.2C.a). Vascular perfusion applied through the convoluted vascularized channel supported tissue viability and maturation for more than two months. PTECs constricted and maintained the tubular architecture during the long-term culture. The 3D fluidic culture enhanced epithelial morphology (higher cell, tissue-like polarized epithelium, presence of cilia on apical side, basement membrane formation on basal side) and functional properties (albumin uptake and megalin expression) of PTEC and proximal tubule tissue (Figure 9.2C.b, C.c, and C.d). Dose-dependent responses to nephrotoxin and immunosuppressive drug were observed in the model, showing its application in high-throughput screening. They also fabricated three-layer tissue construct with proximal tubule located between two perfusable vascular networks (Figure 9.2C.e), demonstrating how bioprinting can be combined with microfluidics for tissue engineering applications (Homan et al. 2016).

Bertassoni et al. (2014) created vascular channels using agarose fiber as a sacrificial template. Agarose gel was printed in fiber form through extrusion in the desired patterns. Then, photocross-linkable hydrogel was added, enclosing the agarose fibers. After the solidification of hydrogel, the agarose templates were removed by applying light vacuum. Perfusable channels with branching structures in submillimeter scale were successfully fabricated within soft hydrogel using this method. A mature endothelium was formed after endothelial cell seeding, and the channel improved the viability of tissue block.

Besides the use of sacrificial materials, the direct removal or ablation of hydrogel matrix in desired channel patterns can be utilized to create hollow channels (Sarig-Nadir et al. 2009; Ilina et al. 2011; Hribar et al. 2015). Hribar et al. (2015) created microchannels within cell-encapsulating collagen hydrogel using near-infrared laser. Collagen hydrogel containing gold nanorods and endothelial cells was prepared, and then a focused near-infrared laser beam was applied to stimulate the nanorods to locally denature the collagen and form hollow channels (Figure 9.2E.a). During postfabrication culture, the adjacent endothelial cells migrated into the hollow channel and form an endothelial monolayer on the inner surface of the channel (Figure 9.2E.b, E.c). In this model, the writing speed and laser power are the important factors to determine the cell viability and channel dimensions. The microchannel was successfully endothelialized without secondary cell seeding procedure in this research, suggesting an alternative solution for endothelialization of microvessels. Ilina et al. (2011) and Sarig-Nadir et al. (2009) created microchannels within hydrogels. The microchannels in their research were designed to serve as guidance channels, but the fabrication methods can be potentially used for constructing vascular channels within 3D matrices.

As a solution to create perfusable tissue matrix, coaxial extrusion printing has been introduced to the tissue engineering field (Yu et al. 2016). The incorporation of coaxial nozzles allows fabricating long-hollow hydrogel filaments by injecting hydrogel precursor through the outer tube and cross-linking agents through inner tube (Figure 9.3A) (Gao et al. 2015). Once the two flows of hydrogel precursor

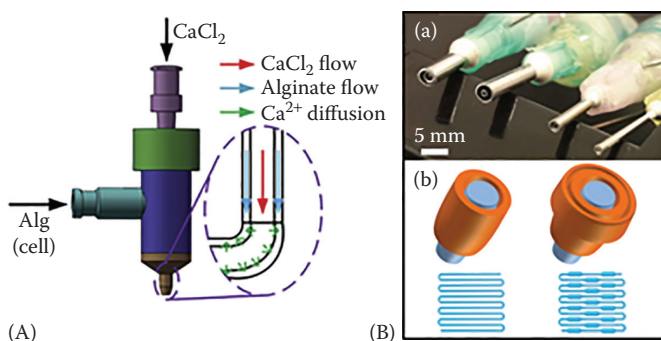
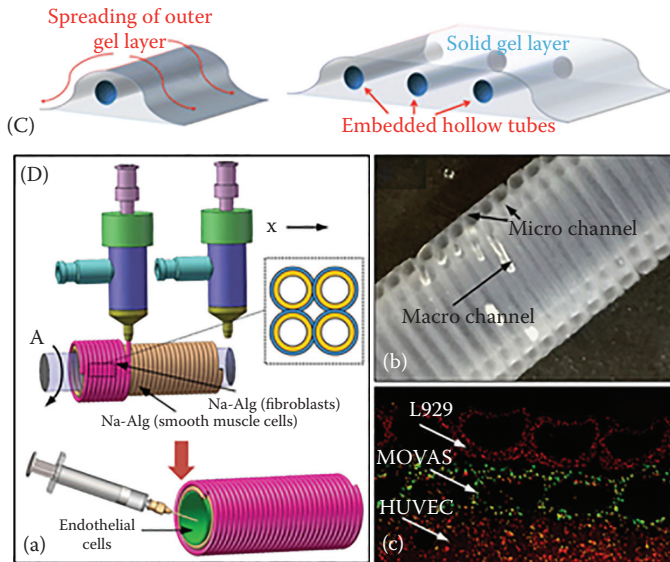


Figure 9.3 Coaxial extrusion bioprinting. (A) Schematics of coaxial nozzle. Alginate filament with a hollow channel was formed by flowing alginate solution through the outer nozzle and calcium chloride solution through the inner nozzle. (Adapted from Gao, Q. et al., *Biomaterials*, doi:10.1016/j.biomaterials.2015.05.031, 2015. With permission.) (B) Multilayered coaxial nozzles shown in (a). (b) Double-layered coaxial nozzle for the fabrication of hollow tubes with constant diameters (left) and trilayered coaxial nozzle for the fabrication of hollow tubes with changeable sizes (right). (Adapted from Jia, W. et al., *Biomaterials*, 106, 58–68, 2016. With permission.) (Continued)



and cross-linking solution contact, the cross-linking agents diffuse into the hydrogel and solidify the inner side of the hydrogel flow. The outer surface of hydrogel flow becomes solidified when the filament is printed into the reservoir bath filled with cross-linking solution.

Just like the filaments printed through a traditional single-nozzle extruder, the hollow filaments can be stacked in desired patterns to create 3D structures. The printed filaments can be fused with adjacent filaments or the hollow filament strand can be encapsulated within other hydrogel to construct a hydrogel mass containing perfusable channels. This approach of using coaxial nozzle does not require additional step of removal process, thus empowering rapid and efficient fabrication of perfusable channels within hydrogel mass. The downsides of the

approach include a lack of ability to create connected bifurcated channels and a risk of collapsing during the fabrication process due to the excessive void spaces.

Multilayered coaxial nozzles (trilayered nozzles) were developed to enable direct extrusion of perfusable tube filaments with varying diameters (Figure 9.3B.a, B.b) (Jia et al. 2016). Using the multilayered coaxial nozzles and two-step cross-linking with blended bioink, Jia et al. created 3D structures with perfusable hollow channels. Alginate part of the blended ink was cross-linked by calcium, and then the photocross-linkable part was polymerized. After complete polymerization, alginate was removed from the printed construct by soaking in ethylenediaminetetraacetic acid (EDTA). Endothelial cells and MSCs embedded in the bioink showed over 70% of cell viability after photocross-linking and EDTA immersion, and successfully proliferated within the hollow tube structures (Jia et al. 2016). Attalla et al. (2016) utilized 3D printer modified with a microfluidic printhead similar to coaxial nozzle to create 3D matrix structure containing hollow tubes. The cross-linking solution bath for full solidification of the printed filament was excluded in their method. Instead, the printed filaments with hydrogel and cross-linking solution were deposited on a substrate, allowing only the inner side of the hydrogel to be cross-linked, whereas the outer gel was slowly solidified to have time to spread out and fill the spaces between hollow tubes (Figure 9.3C).

In another research, the coaxial extruded hollow tube filaments were printed along a rotated rod template to create multilevel vessel structure that consists of macrohollow tube walled with microhollow tubes (Figure 9.3D.a, D.b) (Gao et al. 2017). Two layers of hollow microtubes containing fibroblasts and smooth muscle cells were printed on the rotating rod, and then endothelial cells were seeded on the inner surface (Figure 9.3D.a, D.b). Although the architecture of the multilevel vascular conduit was different from actual vascular network with vascular branches and anastomosis, this approach has the following advantages in tissue vascularization: (a) The multi level vessels may improve transport of nutrient, oxygen, and waste materials; (b) the outer microchannels containing cells may provide initiation points for anastomosis or angiogenesis; and (c) the method can be further developed to recapitulate multilayer multiscale vasculatures (e.g., trilayered arteriole connected to capillary bed) with various matrix and cell types.

9.4 Current issues/challenges and future directions

3D-bioprinting technology is a powerful tool used in numerous areas including tissue engineering, as it enables sophisticated patterning of various biomaterials in 3D space. However, creating large-scale tissues has still been limited due to the architectural complexity and an inability to generate proper vascularization. Although remarkable progress has been made in recapitulating the structural

complexity of native tissue, developing vascularization still remains a major obstacle. Numerous bioprinting techniques have been established to address this issue, and made initial success in vascularization; however further developments are required to address the following challenges in bioprinting vascular networks:

- *Multiscale vascular network*: Millimeter or submillimeter scale of vasculatures has been developed using various bioprinting technologies. There are some successes in creating microvessels; however, the engineering of a complete capillary network is yet to be achieved due to the limitations on spatial resolution and fabrication time. Bridging large vessel with capillaries is also crucial to establish a connected multiscale vascular network. Lee et al. (2014a) and Gao et al. (2017) demonstrated novel approaches to creating multiscale vessel system using vasculogenesis with anastomosis and coaxial printing on a rod template, respectively; however, further developments are required to increase the complexity of vascular network and to eventually control the flow-relevant factors.
- *Multilayer vascular structure*: Blood vessels other than capillary consist of two or more concentric layers with different cell and extracellular matrix (ECM) compositions. This layered structure plays an important role in acquiring sufficient mechanical strengths and hemodynamic functions of the vasculature. A fabrication of larger multilayered vessels (e.g., arteriole, venule, artery, and vein) should be accomplished to recapitulate diverse vascular functions including barrier function, vasodilation, and vasocontraction. Intensive studies in this field will also lead us to develop physiological *in vitro* vascular disease models and to eventually get better understandings about vascular tissue behaviors and vascular diseases.
- *Perfusability*: For macrovessels in millimeter or submillimeter scale, a blockage of vascular perfusion may be caused by collapsing vessel due to poor mechanical properties or rapid degradation of hydrogel and/or by contraction of supporting mural cells. For microvasculatures, endothelial cells often form plexuses with lumen-like structures; however many of them end up being not perfusable. In order to deal with this perfusability issue, substantial efforts need to be made in the field of vascular biology and vascular bioprinting.
- *Bioreactors and long-term culture*: The engineered tissues undergo a long period of culture for further maturation and functional developments. To sustain the tissue viability and support the maturation process, the use of proper bioreactors is indispensable. An incorporation of an improved version of bioreactor that can provide complex flow and

pressure patterns may allow better control and further improvements on the vascular maturation and development process (e.g., branching, angiogenesis, vasculogenesis, thickening, recruitment of perivascular supporting cells).

- *Tissue-specific vessel design*: Every tissue and organ has its unique architectures and composition of cells and matrices. To engineer biomimetic tissues, tissue-specific or organ-specific vascular printing schemes have to be developed. In addition to the fine-tuned fabrication design, cells and matrices that can mimic the targeted tissue vascular system should be provided. Various types of stem cells are good candidates because single-sourced cells can be differentiated into multiple cell lineages and possibly can promote a deposition of ECM that is specific to the targeted tissue or organ.

Developments of novel bioprinting modalities, bioink materials, and bioprinting approaches may add new directions to solve the issues. One example of emerging printing technique for soft materials is 4D printing, in which the printed structure is deformed by stimulus or by the passage of time (Tibbits 2014; Gladman et al. 2016). 4D printing has potential in the generation of complicated 3D vessels out of a simpler form of initial 2D or 3D patterns. It can also provide gradually changing mechanical forces on tissue, which can be utilized to tune mechanical stability or to induce mechanobiological factors. As for biomaterials, pressing issue is to develop biomaterials to achieve desired mechanical, chemical, and biological properties for a specific targeted tissue or organ. For bioprinting, further progress is necessary to develop more biocompatible bioinks that can support (1) high cell viability during and after the printing process and (2) functional development and structural integrity during long-term culture. In addition, the printing ability can be further improved by utilizing viscosity-tunable bioinks and materials that are capable of easy phase transition or multistep phase transitions.

Another way to address the challenges is to combine the current existing techniques. Each bioprinting technologies have its own strengths, which can compensate others' limitations and further can enhance the bioprinting capability. For example, a bioprinting system can be equipped with both extrusion and inkjet nozzle and a photopolymerization platform to effectively fabricate macro and microstructures using a single system. Microfluidic approaches also can be combined with bioprinting technologies, as briefly shown in Attalla et al. (2016); Homan et al. (2016).

All the novel approaches and technologies for bioprinting vascular network will further promote the tissue engineering applications and the clinical translation of engineered tissues by hurdling the obstacles in the field.

References

- Albritton, J. L., and J. S. Miller. 2017. 3D Bioprinting: Improving *in vitro* models of metastasis with heterogeneous tumor microenvironments. *Disease Models & Mechanisms*. doi:10.1242/dmm.025049.
- Attalla, R., C. Ling, and P. Selvagapathy. 2016. Fabrication and characterization of gels with integrated channels using 3D printing with microfluidic nozzle for tissue engineering applications. *Biomedical Microdevices*. doi:10.1007/s10544-016-0042-6.
- Bertassoni, L. E., M. Cecconi, V. Manoharan, M. Nikkhah, J. Hjortnaes, A. L. Cristino, G. Barabaschi et al. 2014. Hydrogel bioprinted microchannel networks for vascularization of tissue engineering constructs. *Lab Chip*. doi:10.1039/C4LC00030G.
- Bhattacharjee, T., S. M. Zehnder, K. G. Rowe, S. Jain, R. M. Nixon, W. G. Sawyer, and T. E. Angelini. 2015. Writing in the granular gel medium. *Science Advances*. doi:10.1126/sciadv.1500655.
- Christensen, K., C. Xu, W. Chai, Z. Zhang, J. Fu, and Y. Huang. 2015. Freeform inkjet printing of cellular structures with bifurcations. *Biotechnology and Bioengineering*. doi:10.1002/bit.25501.
- Datta, P., B. Ayan, and I. T. Ozbolat. 2017. Acta biomaterialia bioprinting for vascular and vascularized tissue biofabrication. *Acta Biomaterialia* 51: 1–20. doi:10.1016/j.actbio.2017.01.035.
- Gao, Q., Y. He, J. Fu, A. Liu, and L. Ma. 2015. Coaxial nozzle-assisted 3D bioprinting with built-in microchannels for nutrients delivery. *Biomaterials*. doi:10.1016/j.biomaterials.2015.05.031.
- Gao, Q., Z. Liu, Z. Lin, J. Qiu, Y. Liu, A. Liu, Y. Wang et al. 2017. 3D bioprinting of vessel-like structures with multilevel fluidic channels. *ACS Biomaterials Science and Engineering*. doi:10.1021/acsbomaterials.6b00643.
- Gebinoga, M., J. Katzmann, U. Fernekorn, J. Hampl, F. Weise, M. Klett, A. Löffert, T. A. Klar, and A. Schober. 2013. Multi-photon structuring of native polymers: A case study for structuring natural proteins. *Engineering in Life Sciences*. doi:10.1002/elsc.201200152.
- Gladman, A., A. E., Matsumoto, R. G. Nuzzo, L. Mahadevan, and J. A. Lewis. 2016. Biomimetic 4D printing. *Nature Materials*. doi:10.1038/nmat4544.
- Highley, C. B., C. B. Rodell, and J. A. Burdick. 2015. Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels. *Advanced Materials*. doi:10.1002/adma.201501234.
- Hinton, T. J., Q. Jallerat, R. N. Palchesko, J. H. Park, M. S. Grodzicki, H.-J. Shue, M. H. Ramadan, A. R. Hudson, and A. W. Feinberg. 2015. Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances*. doi:10.1126/sciadv.1500758.
- Homan, K. A., D. B. Kolesky, M. A. Skylar-Scott, J. Herrmann, H. Obuobi, A. Moisan, and J. A. Lewis. 2016. Bioprinting of 3D convoluted renal proximal tubules on perfusable chips. *Scientific Reports*. doi:10.1038/srep34845.
- Hribar, K. C., K. Meggs, J. Liu, W. Zhu, X. Qu, and S. Chen. 2015. Three-dimensional direct cell patterning in collagen hydrogels with near-infrared femtosecond laser. *Scientific Reports*. doi:10.1038/srep17203.
- Hribar, K. C., P. Soman, J. Warner, P. Chung, and S. Chen. 2014. Light-assisted direct-write of 3D functional biomaterials. *Lab Chip*. doi:10.1039/C3LC50634G.
- Huang, T. Q., X. Qu, J. Liu, and S. Chen. 2014. 3D printing of biomimetic microstructures for cancer cell migration. *Biomedical Microdevices*. doi:10.1007/s10544-013-9812-6.
- Ilina, O., G. J. Bakker, A. Vasaturo, R. M. Hoffman, and P. Friedl. 2011. Two-photon laser-generated microtracks in 3D collagen lattices: Principles of MMP-dependent and -independent collective cancer cell invasion. *Physical Biology* 8 (2): 029501–029501.

- Jia, W., P. S. Gungor-Ozkerim, Y. S. Zhang, K. Yue, K. Zhu, W. Liu, Q. Pi et al. 2016. Direct 3D bioprinting of perfusable vascular constructs using a blend bioink. *Biomaterials* 106: 58–68. doi:10.1016/j.biomaterials.2016.07.038.
- Kaully, T., K. Kaufman-Francis, A. Lesman, and S. Levenberg. 2009. Vascularization—The conduit to viable engineered tissues. *Tissue Engineering Part B: Reviews* 15 (2): 159–169. doi:10.1089/ten.teb.2008.0193.
- Khademhosseini, A., and R. Langer. 2016. A decade of progress in tissue engineering. *Nature Protocols* 11 (10): 1775–1781. doi:10.1038/nprot.2016.123.
- Kolesky, D. B., K. A. Homan, M. A. Skylar-Scott, and J. A. Lewis. 2016. Three-dimensional bioprinting of thick vascularized tissues. *Proceedings of the National Academy of Sciences the United States of America*. doi:10.1073/pnas.1521342113.
- Kolesky, D. B., R. L. Truby, A. S. Gladman, T. A. Busbee, K. A. Homan, and J. A. Lewis. 2014. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials* 26 (19): 3124–3130. doi:10.1002/adma.201305506.
- Lee, V. K., D. Y. Kim, H. Ngo, Y. Lee, L. Seo, S. S. Yoo, P. A. Vincent, and G. Dai. 2014b. Creating perfused functional vascular channels using 3D bio-printing technology. *Biomaterials* 35 (28): 8092–8102. doi:10.1016/j.biomaterials.2014.05.083.
- Lee, V. K., A. M. Lanzi, H. Ngo, S. S. Yoo, P. A. Vincent, and G. Dai. 2014a. Generation of multi-scale vascular network system within 3D hydrogel using 3D bio-printing technology. *Cellular and Molecular Bioengineering* 7 (3): 460–472. doi:10.1007/s12195-014-0340-0.
- Lovett, M., K. Lee, A. Edwards, and D. L. Kaplan. 2009. Vascularization strategies for tissue engineering. *Tissue Engineering Part B Reviews* 15 (3): 353–370. doi:10.1089/ten.TEB.2009.0085.
- Meyer, W., S. Engelhardt, E. Novosel, B. Elling, M. Wegener, and H. Krüger. 2012. Soft polymers for building up small and smallest blood supplying systems by stereolithography. *Journal of Functional Biomaterials* 3 (4): 257–268. doi:10.3390/jfb3020257.
- Miller, J. S., K. R. Stevens, M. T. Yang, B. M. Baker, D. T. Nguyen, D. M. Cohen, E. Toro et al. 2012. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials* 11 (9): 768–774. doi:10.1038/nmat3357.
- Nahmias, Y., R. E. Schwartz, C. M. Verfaillie, and D. J. Odde. 2005. Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnology and Bioengineering* 92 (2). doi:10.1002/bit.20585.
- Nerem, R. M., and D. Seliktar. 2001. Vascular Tissue Engineering. *Annual Review of Biomedical Engineering* 3 (1): 225–243. doi:10.1146/annurev.bioeng.3.1.225.
- Norotte, C., F. S. Marga, L. E. Niklason, and G. Forgacs. 2009. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials* 30 (30): 5910–5917. doi:10.1016/j.biomaterials.2009.06.034.
- Ozolat, I. T., K. K. Moncal, and H. Gudapati. 2017. Evaluation of bioprinter technologies. *Additive Manufacturing*. doi:10.1016/j.addma.2016.10.003.
- Pashneh-Tala, S., S. MacNeil, and F. Claeysens. 2015. The tissue-engineered vascular graft—past, present, and future. *Tissue Engineering Part B: Reviews*. doi:10.1089/ten.teb.2015.0100.
- Phelps, E. A., and A. J. Garcia. 2010. Engineering more than a cell: Vascularization strategies in tissue engineering. *Current Opinion in Biotechnology* 21 (5): 704–709. doi:10.1016/j.copbio.2010.06.005.
- Rouwkema, J., N. C. Rivron, and C. A. van Blitterswijk. 2008. Vascularization in tissue engineering. *Trends in Biotechnology* 26 (8): 434–441. doi:10.1016/j.tibtech.2008.04.009.
- Sarig-Nadir, O., N. Livnat, R. Zajdman, S. Shoham, and D. Seliktar. 2009. Laser photoablation of guidance microchannels into hydrogels directs cell growth in three dimensions. *Biophysical Journal* 96 (11): 4743–4752. doi:10.1016/j.bpj.2009.03.019.

- Schiele, N. R., D. T. Corr, Y. Huang, N. A. Raof, Y. Xie, and D. B. Chrisey. 2010. Laser-based direct-write techniques for cell printing. *Biofabrication* 2 (3): 32001. doi:10.1088/1758-5082/2/3/032001.
- Suri, S., L. H. Han, W. Zhang, A. Singh, S. Chen, and C. E. Schmidt. 2011. Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering. *Biomedical Microdevices*. doi:10.1007/s10544-011-9568-9.
- Tan, Y., D. J. Richards, T. C. Trusk, R. P. Visconti, M. J. Yost, M. S. Kindy, C. J. Drake, W. S. Argraves, R. R. Markwald, and Y. Mei. 2014. 3D printing facilitated scaffold-free tissue unit fabrication. *Biofabrication* 6 (2): 24111. doi:10.1088/1758-5082/6/2/024111.
- Tibbits, S. 2014. 4D printing: Multi-material shape change. *Architectural Design*. doi:10.1002/ad.1710.
- Xiong, R., Z. Zhang, W. Chai, Y. Huang, and D. B. Chrisey. 2015. Freeform drop-on-demand laser printing of 3D alginate and cellular constructs. *Biofabrication*. doi:10.1088/1758-5090/7/4/045011.
- Xu, C., W. Chai, Y. Huang, R. R. Markwald, and S. Carolina. 2012. Scaffold-free inkjet printing of three-dimensional zigzag cellular tubes. *Biotechnology and Bioengineering* 109 (12): 3152–3160. doi:10.1002/bit.24591.
- Xu, C., Z. Zhang, K. Christensen, Y. Huang, J. Fu, and R. R. Markwald. 2014. Freeform vertical and horizontal fabrication of alginate-based vascular-like tubular constructs using inkjetting. *Journal of Manufacturing Science and Engineering*. doi:10.1115/1.4028578.
- Yu, Y., K. K. Moncal, J. Li, W. Peng, I. Rivero, J. A. Martin, and I. T. Ozbolat. 2016. Three-dimensional bioprinting using self-assembling scalable scaffold-free ‘tissue strands’ as a new bioink. *Scientific Reports*. doi:10.1038/srep28714.
- Zhang, A. P., X. Qu, P. Soman, K. C. Hribar, J. W. Lee, S. Chen, and S. He. 2012. Rapid fabrication of complex 3D extracellular microenvironments by dynamic optical projection stereolithography. *Advanced Materials*. doi:10.1002/adma.201202024.
- Zhu, W., X. Ma, M. Gou, D. Mei, K. Zhang, and S. Chen. 2016. 3D printing of functional biomaterials for tissue engineering. *Current Opinion in Biotechnology*. doi:10.1016/j.copbio.2016.03.014.



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Chapter 10 Bioprinting of living aortic valve

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and J.T. Butcher

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10.1 Introduction

The human heart consists of four heart valves, all of which direct unidirectional blood flow through the cardiovascular system via opening and closing of compliant valvular leaflets. Among the four valves, the aortic valve—situated between

the left ventricle and the aorta—experiences the most challenging hemodynamic environment and is also the primary valve that becomes diseased. Valvular heart disease (VHD) can be characterized by damage or defect to the valves, mainly either through leaflet stenosis (stiffening of leaflets) or insufficiency (regurgitation of blood from retracted leaflets) (Nishimura 2002). The heart compensates the reduced blood flow by enlarging its muscles to pump more blood, thereby reducing its elasticity, which can lead to heart failure.

VHD is among the major forms of cardiovascular diseases. The prevalence of any valve disease adjusted to the entire U.S. population is 2.5% (approximately 8 million people), with prevalence increasing with age (>65 years)—the majority of which were the aortic valves (68%) (Benjamin et al. 2017). In addition, 1%–2% of all live births are affected by congenital heart disease, many of which affect the heart valves, leading to 44,000 cases annually (Go et al. 2013). In total, the total inflation-adjusted cost in 2013 was U.S.\$5.264 billion, an increase from previous years (Benjamin et al. 2017), and it is estimated that heart valve replacement surgeries to triple by 2050 (Yacoub and Takkenberg 2005).

On a global scale, valve disease is mainly attributed to rheumatic heart disease, which has shown an increasing trend in the Americas, Africa, Eastern Mediterranean, Western Pacific, and Southeast Asia (Benjamin et al. 2017). The population affected is largely skewed toward pediatric and young adults and in populations with lower income, resulting in a prevalence of 15.6 million and 233,000 deaths annually (Carapetis et al. 2005).

Current treatment options—heart valve replacement—are generally viable for the elderly, where the implants will last longer than the patient's lifespan. However, pediatric patients and young adults face other risks associated with these options, including multiple resizing surgeries. The inadequacy of the replacement valves stems from the inability for the prosthetic valve to integrate with the patient to repair, regenerate, and grow. The lack of a living replacement, combined with the number of patients affected, motivates researchers to fabricate tissue-engineered heart valves that address the major challenges of current valve replacement options (Vahanian et al. 2012).

This chapter will provide an overview of aortic heart valve anatomy and physiology along with its cellular compositions to highlight engineering considerations for fabricating tissue-engineered heart valves. Current treatment options and limitations will be summarized and evaluated. Next, this chapter will dive into reviewing current tissue engineering (TE) strategies for fabricating heart valves and highlight 3D bioprinting as an upcoming strategy for overcoming persisting challenges.

10.2 A multiscale overview of the aortic heart valve

10.2.1 The aortic heart valve

At the organ level, the aortic heart valve is composed of three compliant cusps (also known as leaflets) attached to the aortic root wall ([Figure 10.1a](#)) (Charitos and Sievers 2013). Each leaflet is identified on the basis of the location within the root: the left and right cusps, and the noncoronary cusp. The individual structures work synergistically to direct blood flow out of the heart (i.e. fully opening of the leaflets) while minimizing regurgitation (i.e. fully closing of the leaflets). The rapid valvular motions—an estimated 30 million times per year (Butcher et al. 2011)—produce complex hemodynamics with the surrounding blood, which can induce large shear forces on the valve surfaces (Sacks and Yoganathan 2007). Thickening and calcification of the valve can lead to altered flow patterns, which can exacerbate the disease (Bäck et al. 2013).

10.2.2 Valve leaflets

The leaflets consist of a complex matrix architecture that can be split into three distinct layers: (1) fibrosa, (2) spongiosa, and (3) ventricularis ([Figure 10.1b](#)). Each layer is composed of different cell types and structural components and therefore serves a distinct purpose and giving rise to the anisotropic biomechanical properties of the leaflets (Latif et al. 2005). The fibrosa faces toward the ascending aorta and is the load-bearing layer, constituting approximately 41% of the total leaflet thickness (Stella and Sacks 2007). It is composed of densely packed collagen fibers aligned circumferentially, which exhibits strong mechanical strength on closure to bear the higher diastolic load (Balguind et al. 2007; Thubrikar et al. 1980). The spongiosa is a glycosaminoglycan- and proteoglycan-rich zone situated between the fibrosa and ventricularis and takes up about 30% of the thickness. The hydroscopic layer provides compressive resistance during diastole (leaflet coaptation), a buffer or lubrication zone between the fibrosa and ventricularis during systole (leaflet flexure), and a dampening effect to reduce the leaflet flutter (MacGrogan et al. 2014; Eckert et al. 2013; Buchanan and Sacks 2014). Finally, the remaining 29% of the leaflet is the ventricularis, which faces toward the left ventricle. This layer is mainly composed of collagen and elastin sheets aligned radially, thus contributing to the radial strength of the leaflet (Vesely 1998; Vesely and Noseworthy 1992; Sacks and Yoganathan 2007). Together, the complex anisotropic leaflets can withstand the estimated physiological loads between 200–400 kPa and peak Green strains of 0.264 and 1.109 in the circumferential and radial directions, respectively (Thubrikar et al. 1980; Lee et al. 1984; Stella and Sacks 2007; Billiar and Sacks 1999).

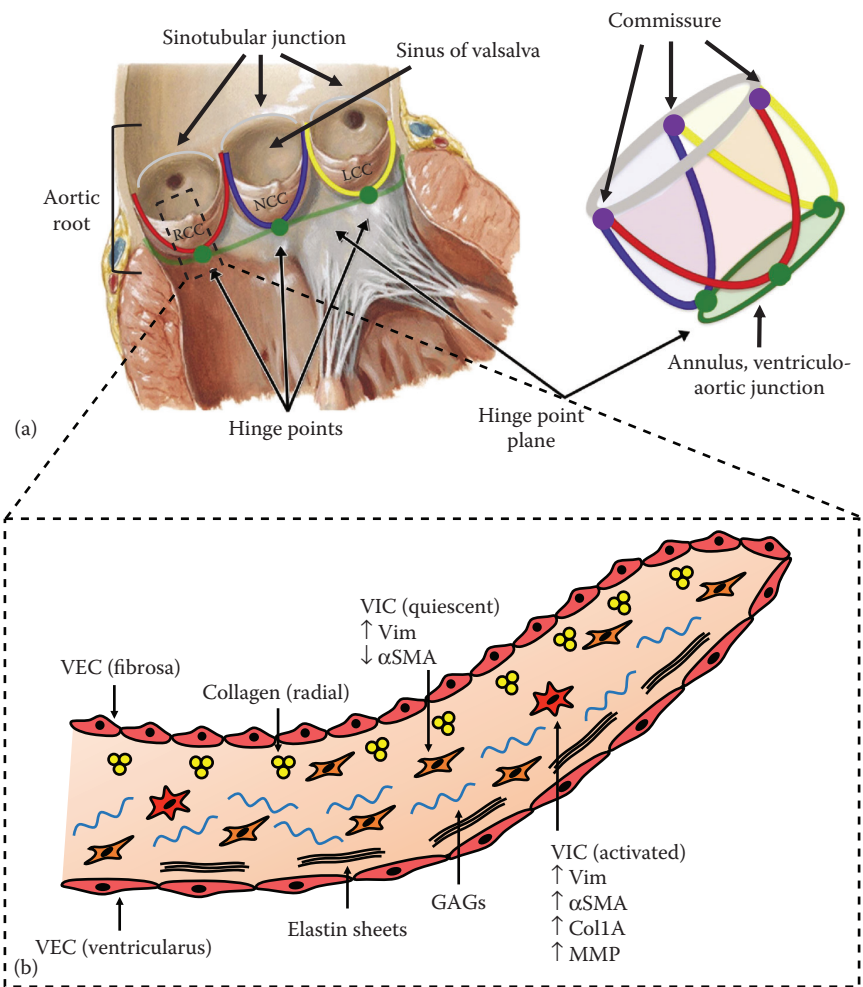


Figure 10.1 Overview of the macro- and microscopic aortic heart valve anatomy. (a) Schematic of an open aortic heart valve. Three compliant leaflets or cusps are located within the sinus bulb and attached at the base of the aortic root (annulus or ventriculo–aortic junction). (Adapted from Kasel, A.M. et al., *JACC Cardiovasc. Imaging*, 6, 249–262, 2013. With permission.) (b) Schematic of a radially sliced valve leaflet. VEC line the fibrosa and ventricularis slide, whereas VIC (quiescent and activated) are spread throughout the interstitium. Major ECM components include collagen in the fibrosa, GAGs in the spongiosa, and elastin in the ventricularis. GAG = glycosaminoglycans, LCC = left coronary cusp, NCC = noncoronary cusp, RCC = right coronary cusp, VEC = valve endothelial cell, VIC = valve interstitial cell.

10.2.3 Valve root

The aortic root wall encloses the leaflets and comprises a variety of entities crucial to the function of the organ (Charitos and Sievers 2013; Ho 2009). The sinuses of Valsalva are curved dilations occurring immediately anterior of each leaflet, making the root wall an inverted bulb shape. The left and right sinuses give rise to the left and right coronary arteries, respectively. The cusps are attached to the base of the root where they form a crown shape with the annulus, which is a fibrous ring that is considered a transition point between the ventricle and aortic root. The leaflets are joined together at commissural points, which are connected to the sinotubular junction, the distal ring portion of the sinuses. The histological composition of the aortic root, although not extensively studied, is similar to the ascending aorta and other large arteries, consisting of intimal, medial, and adventitial layers (Ho 2009; Butcher et al. 2011). The intimal and medial layers contain mainly of elastin, whereas the adventitial layer had higher levels of collagen (Iliopoulos et al. 2013; Azadani et al. 2012a, 2012b). Although similar in histology, the sinus displayed larger tissue stiffness in both the longitudinal and circumferential directions. In addition, the biaxial tensile testing showed no significant differences in stiffness between longitudinal and circumferential directions (approximately 3.5 MPa) (Azadani et al. 2012a). Overall, the entire root structure is stiffer than the leaflets.

10.2.4 Valve leaflet cells

At the cellular level, there are only a few main cell types residing in the aortic leaflet and root but with varying degrees of phenotypes. In the aortic leaflet, valvular endothelial cells (VEC) line the fibrosa and ventricularis in a confluent monolayer. As opposed to other endothelial cells in blood vessels, aortic VEC aligns perpendicular to flow rather than parallel to the flow (Butcher and Nerem 2006). The pulsatile nature of blood flow induces dynamic leaflet deformations, and the shear environment changes with the cardiac cycle (Dagum et al. 1999; Sacks and Yoganathan 2007). Fibrosa VEC experiences oscillatory shear stresses during systole compared to the laminar shear stresses on the ventricularis side (Balachandran et al. 2011). During diastole, the back pressure from the aorta forces the leaflets to close and coapt, which stretches the leaflets from the fibrosa. These external environments are sensed by VEC in a side-specific manner, which give them side-specific properties and phenotype (Weinberg et al. 2010; Miragoli et al. 2014). In addition, VEC play an important role in signal transduction to the interstitial cells initiated by physical (e.g. shear, deformation) (Butcher et al. 2011) or biochemical (e.g. oxidative stress, inflammation) stimuli (Butcher and Markwald 2007). Similar to other endothelial cells, VEC provide anticoagulant properties (Kasimir et al. 2005). Together, these properties of VEC play a role in homeostasis and hemostasis on the valve.

The valve interstitial cells (VIC) are the mesenchymal cells and most abundant cells within the leaflets. Although they are a single cell type, they display a dynamic and continuous spectrum of phenotypes, unlike many other types of mesenchymal cells in other organ systems (Liu et al. 2007). Under normal circumstances in healthy, mature valves, majority of the cells are in the quiescent state (VIM+, SMA-). However, VIC can become activated to a myofibroblastic phenotype (VIM+, SMA+) in certain biochemical environments (e.g. inflammation) or to help basal ECM turnover (Pho et al. 2008). This activated VIC can further differentiate toward an osteoblastic-like lineage (osteocalcin+, Runx2+), which can lead to matrix calcification (Farrar et al. 2016; Yip et al. 2009). Other phenotypes, although more uncommon, include progenitor (SSEA-4+ or ABCG2+/NG2+) (Wang et al. 2013) and smooth muscle (calponin+, MYH11+). Researchers are still elucidating the roles of each phenotype in relation to valve homeostasis, but a general consensus supports the role of VIC on valve maintenance and dysfunction (Taylor et al. 2003; Chen et al. 2009; Mahler et al. 2013). VIC isolated from different valve cusps may have inherently different predisposition to calcification, notably VIC derived from the noncoronary cusp displayed more calcific markers than VIC derived from the coronary cusps (Masjedi et al. 2016).

10.2.5 Root wall cells

Similar to the valves, the aortic root—specifically the sinus in this context—comprises a heterogeneous population of cells, including aortic endothelial cells lining the root and smooth muscle cells (SMC) and fibroblasts in the bulk of the tissue (López-Mínguez et al. 2006). Although the cells within the aortic root are not well characterized and studied, they may act similarly to cells found in the ascending aorta, although there may be differences in phenotype within the sinus due to the different mechanical forces present (Bäck et al. 2013). The endothelial cells provide an anticoagulant barrier to regulate hemostasis and may secrete factors to regulate vasoconstriction and vasodilation. Unlike VEC, these endothelial cells align parallel to blood flow, which may suggest that these cells respond differently to the shear environments and other biochemical cues (Butcher and Nerem 2006; Armstrong and Bischoff 2004). SMC may be able to sense biomechanical factors through tissue deformation from the pulsatile nature of the cardiac cycle, but it is not certain to what extent the SMC behave similarly to those found in large arteries.

10.3 Engineering considerations for TEHV

The complexity of the aortic heart valve leads to multiscale engineering considerations for achieving success (Cheung et al. 2015). At the organ level, the valve must fully open during systole and coapt during diastole without leaflet retraction.

The leaflets and root should match the native tissue stiffness within the physiological strain ranges or be robust enough to withstand the hemodynamic conditions. Too compliant of tissue may result in premature deterioration, whereas too stiff of an environment could cause pathological responses. On implantation, the tissue engineered heart valves (TEHV) should not invoke thrombosis. Immunogenic response should either be harnessed to induce regeneration or avoided to reduce acute immune response. The scaffold should facilitate cellular infiltration and promote matrix remodeling and neotissue deposition before it fully biodegrades. Regenerated tissue should integrate with the host such that the TEHV grows as the host grows. Ideally, the deposited tissue will recapitulate the anisotropic trilayer structure of the valve and populated by VEC and quiescent or activated VIC. Other design considerations may include the ease and reproducibility of fabrication, cost of synthesis, cell source (if any), type of product (off-the-shelf or patient-specific), and any regulatory hurdles. Along with these engineering considerations, the fabricated tissue or scaffold should comply with the ISO 5840 standard for cardiovascular implants, specifically for cardiac valve prostheses.

10.4 Overview of current options for aortic valve replacement

10.4.1 Mechanical heart valves

Mechanical heart valves (MHV) remain the most robust option to date. There are three main designs of MHV—(1) monoleaflet, (2) bileaflet, and (3) caged ball valves—but the majority used clinically are bileaflet mechanical valves ([Figure 10.2a](#)) (Pibarot and Dumesnil 2009). The mechanical prosthetics show relatively good short- and long-term outcomes in some elderly patient populations, showing low risk of reoperation and low incidence rates of valve-related complications (Okamoto et al. 2016; Glaser et al. 2015). In addition, in some populations of pediatric and young adult populations, MHV can offer an adequate treatment option, with some long-term (>10 years) survival rates ranging between 60%–70% (Songur et al. 2014; Misawa 2014; Arnold et al. 2008).

Despite being a durable option for patients, there are serious complications and lifestyle limitations for patients with MHV (Misawa 2014). Patients are required to take life-long anticoagulation medication to manage and reduce risks of thromboembolism caused by blood clots formed due to shearing of the blood cells; however, this medication can also cause hemorrhage (Labaf et al. 2016). In addition, pediatric and young patients must consider additional surgeries for valve resizing (Masuda et al. 2008).

Although innovation for mechanical heart valves has slowed down in the past 25 years, there has been progress in research on anticoagulant-independent



Figure 10.2 Highlights of selected current valve prostheses in the market. (a) Mechanical valves (left to right): St. Jude SJM Regent™, Medtronic Open Pivot™, Cryolife On-X®. (b) Bioprosthetic valves (left to right): St. Jude Trifecta, Medtronic Freestyle Aortic Root, Carpentier–Edwards PERIMOUNT. (c) Transcatheter valves (left to right): Medtronic Evolut TAVR, Edwards SAPIEN. (From <http://www.sjm.com>; <http://www.medtronic.com> and <http://www.edwards.com>.)

mechanical valves (Chaux et al. 2016). We have shown that surfaces with microfabricated trenches can reduce shear stresses to a tolerable level such that endothelial cells stay adhered and formed a mature monolayer that expressed anti-coagulant profiles and reduced platelet adhesion (Frendl et al. 2014). Other modification methods may include using plasma-surface modification to immobilize antithrombotic molecules (Chu et al. 2002). Interestingly, one group has developed leaflets composed of nondegradable nitinol mesh scaffold attached to a stent (Alavi et al. 2017). More recently, the Swiss-made Lapeyre–Triflo Furtiva® prosthetic valve incorporated a trileaflet design in a mechanical prosthetic, which has similar hemodynamics as a tissue valve and has shown to be resistant to thrombosis and structural failure (Gallegos et al. 2006; Reineke et al. 2016). Further research on surface modification or biofunctionalization of MHV may provide a promising biohybrid approach for mechanical heart valves.

10.4.2 Bioprosthetic heart valves

Bioprosthetics, unlike mechanical heart valves, are derived from human (homograft) or animal (xenograft) tissues (Figure 10.2b). Homografts are derived from cryopreserved cadaveric valves, and autograft uses the patient's own pulmonary valve in the aortic position. In both cases, the quantity is severely limited. Instead, xenografts are often used, which are usually derived from porcine valves or bovine pericardium (Pibarot and Dumesnil 2009). These valves are treated with a variety of methods to reduce or prevent immunogenicity and calcification via glutaraldehyde cross-linking, antimineralization steps, and high–low pressures (Singhal et al. 2013). Bioprosthetics are fabricated in a few form factors, including stented, stentless, and self-expanding stent (Figure 10.2c). The latter form factor is the only type of valve that can be implanted via the minimally invasive technique transcatheter aortic valve replacement/implantation (TAVR/TAVI).

To date, many valve replacement operations use bioprosthetics over mechanical valves, especially for elderly adults. One major advantage is the recapitulation of native valve kinematics and hemodynamic characteristics. There were no significant differences in 15-year survival or stroke rates (Chiang et al. 2014). Bioprosthetics have low risks for thromboembolism, so life-long anticoagulation medication is not needed. Although there is a higher rate of reoperation, there are lower bleeding rates (Hammermeister et al. 2000; Silberman et al. 2008; Chiang et al. 2014). However, in the case of children and younger patients, lower survival rates and freedom from explantation were observed (Welke et al. 2011).

10.4.3 Ross procedure

The Ross procedure, also known as the pulmonary autograph, replaces the diseased aortic valve with the healthy pulmonary valve. Generally, a bioprosthetic will be placed in the less hemodynamically demanding pulmonary position. This procedure has shown great potential due to enhanced hemodynamic and kinematic performance and lack of anticoagulation therapy (Stelzer 2011). However, the success of this procedure biases the adult population (Sharabiani et al. 2016). One long-term study of the Ross procedure in adults showed a 90.7% 10-year survival rate and high freedom from autograph and pulmonary graft reoperations up to 10 years (Karaskov et al. 2016). However, in younger patients (median age of 4.8 years) had a lower rate of freedom from RVOT intervention, dropping to 59% at 15 years (Karaskov et al. 2016). Similarly, the performance of bioprosthetics in the pulmonary position is dependent on age. In a recent study, bioprosthetics implanted in the pulmonary position in patients with congenital heart disease showed excellent short-term outcomes, but children showed a five-fold greater risk of reintervention than adults (Nomoto et al. 2016). Despite the successes of the operation, the operation turns a one-valve procedure into two

valves, increasing operational risks and complexity. There are still concerns about the growth and remodeling potential of the pulmonary autograft in the aortic position. Furthermore, there are still concerns about using bioprosthetics in the pulmonary position, particularly in younger patients with increased rate of reoperations (Kallio et al. 2015).

10.5 Current engineering strategies for whole valve fabrication

Despite the current available options and their relative successes, each of these replacement options are suboptimal in children and require other replacement options (Etnel et al. 2016). In a classical TE approach, a biocompatible scaffold is fabricated on which autologous cells are seeded. The cells are then able to grow and remodel the scaffold, which can be accelerated in a bioreactor to mimic physiological conditions. The conditioned and mature tissue can then be implanted back into the patient. Similarly, for engineering heart valves, a similar approach is used with some exceptions. There are numerous fabrication techniques and materials used to produce a TEHV scaffold, and we will highlight the predominant techniques used for current research. An overview of current strategies can be found in [Table 10.1](#).

10.5.1 Decellularization

Decellularization is an attractive technique to produce an anatomical valve that can support growth. This method removes all cellular content within the tissue but leaves the ECM structure and protein relatively intact. Thus, these valves are similar to bioprosthetics in their hemodynamic performance (Konertz et al. 2005), but differ in their growth potential because decellularized valves are not chemically fixed and can therefore grow and remodel with the patient (Dohmen et al. 2006a). Theoretically, decellularization can reduce acute immune response while retaining structural integrity and bioactivity of a whole valve (Kneib et al. 2012; Bloch et al. 2011). Specifically, human donor valves can be decellularized, but the clear majority are animal (e.g. porcine) derived due to the high availability of the source tissue (Neumann et al. 2014).

The process of decellularizing a tissue can vary, generally depending on its size and architecture. Common techniques used include physical agitation and disruption (e.g. freeze–thaw), chemical treatment (e.g. detergents), and biological agents (e.g. enzymes) (Crapo et al. 2011). The Badylak group proposed a quantitative criteria to ensure proper tissue decellularization: The tissue shall contain (1) < 50 ng dsDNA/mg ECM (dry weight), (2) < 200 bp DNA fragment lengths, and (3) no nuclear material in histological samples (e.g. H&E, DAPI) (Crapo et al. 2011).

Table 10.1 Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2000	Decellularization	Ovine pulmonary valve		Whole valve	Ovine myofibroblast and endothelial cells	<i>In vivo</i> sheep implantation (2 weeks)	1. Showed histological restitution of tissue and endothelium 2. Correct phenotypic markers appeared 3. Histological signs of inflammation	Steinboff et al. (2000)
2000	Molding	Nonwoven polyglycolic-acid (PGA) mesh coated with poly-4-hydroxybutyrate (P4HB)		Whole valve	Autologous ovine myofibroblast and endothelial cells	<i>In vitro</i> bioreactor conditioning (14 days); <i>in vivo</i> lamb implantation (20 weeks)	1. Mobile, functioning leaflets 2. No stenosis, thrombosis, or aneurysm 3. Showed increase ECM and DNA production, comparable to native 4. Complete polymer degradation	Hoerstrup et al. (2000)
2000	Molding	Polyhydroxyalkanoate (PHO) casted onto aluminum valve cast		Whole valve	Initial seeding with ovine carotid artery vascular cells then with venous endothelial cells	Implanted into pulmonary position of lamb	Minimal regurgitation, low valvular gradients No thrombus formation Layered tissue formation and ECM (GAG, collagen) deposition	Sodian et al. (2000)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2007	Molding	Fibrin		Whole valve	Ovine carotid artery SMCs and fibroblasts	Bioreactor conditioning for 12 days	Conditioned samples revealed ECM protein deposition (collagen I and III, fibronectin, laminin, chondroitin sulfate)	Flanagan et al. (2007)
2010	<i>In vivo</i> engineering, molded stamp	Polyurethane mesh wrapped around silicone valve-shaped mold, tissue membrane as leaflets		Whole valve	N/A	<i>In vivo</i> synthesis in rabbit subcutaneous pouch (month); <i>in vitro</i> characterization in bioreactor	Fibrous tissue formation on mesh; Proper opening and closing of leaflets	Yamanami et al. (2010)
2012	Decellularization	Porcine pulmonary valves conjugated with CD133		Whole valve	Porcine endothelial progenitor cell-derived endothelial cells	<i>In vivo</i> implantation in sheep in pulmonary position (3 months)	vWF+ endothelial cells-lined surface; Increased interstitial cells and collagen content; No calcification or thrombosis observed	Jordan et al. (2012)
2012	Molding	Polyglycolic-acid (PGA) coated with poly-4-hydroxybutyrate (P4HB) and stitched to stent		Self-expandable stented valve	Ovine amniotic fluid cells	Implanted into fetal ovine pulmonary position in-utero (1 week)	High acute survival rate; Functional valve without thrombosis; ECM deposition on TEHV	Weber et al. (2012)
2012	Electrospinning	Poly(ester urethane) urea (PEUU)		Tissue scaffold	N/A	Mechanical characterization	Increasing fiber intersection density lowered bending moduli; Stiff secondary fiber yielded higher bending moduli	Amoroso et al. (2012)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2012	Electrospinning	Poly(glycerol sebacate): poly(caprolactone) (PGS:PCL)		Tissue scaffold	Human VICs	<i>In vitro</i> characterization of scaffold	VIC aligned along anisotropic axes; Production of collagen, vimentin, smooth muscle actin genes; Mechanical properties mimicked native valves	Masoumi et al. (2014)
2012	3D bioprinting	Poly(ethylene glycol)-diacrylate (PEGDA)		Whole valve	Porcine aortic VICs	<i>In vitro</i> static culture (21 days)	Larger valves showed higher shape fidelity; High cell biocompatibility	Hockaday et al. (2012)
2012	Molding, decellularization	Polyglycolic-acid (PGA) coated with poly-4-hydroxybutyrate (P4HB) and stitched to stent wires	Initially seeded with vascular cells then decellularized and reseeded with MSCs	Self-expandable stented valve	Ovine vascular cells, ovine MSCs	<i>In vitro</i> bioreactor conditioning	Valve fully opened and closed; Decellularization did not alter ECM composition or mechanical strength	Dijkman et al. (2012)
2013	Decellularization	Porcine and ovine aortic roots		Whole valve	N/A	<i>In vivo</i> in abdominal aorta of pigs; <i>in vivo</i> in RVOT of juvenile sheep (6 months)	Cellular repopulation of scaffold, with evidence for macrophage-induced regeneration; Calcification observed in two valves	Paniagua Gutierrez et al. (2015)
2013	Decellularization	Rat aortic roots		Whole valve	N/A	<i>In vitro</i> architecture characterization and reseeding; <i>in vivo</i> implantation (14 days)	Preservation of ECM after decellularization; Cells successfully reseeded onto valve; Increased peak pressure and valve stiffness; No signs of immunogenicity	Friedrich et al. (2014)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2013	3D bioprinting	Alginate/gelatin		Whole valve	Porcine aortic VICs and SMCs	<i>In vitro</i> static culture (7 days)	Scaffolds with cells increased scaffold modulus, UTS, and peak strain; SMC expressed elevated smooth muscle actin; VIC expressed elevated vimentin	Duan et al. (2013)
2013	3D bioprinting	Methacrylated hyaluronic acid (MeHA)/methacrylated gelatin (MeGel)		Valve leaflet	Human aortic VICs	<i>In vitro</i> static culture (14 days)	Enhanced cell spreading, metabolic activity, migration; Increased GAG deposition; Maintained VIC phenotype	Duan et al. (2013)
2013	Molding, decellularization	Fibrin	Seeded construct conditioned in bioreactor first then decellularized	Tubular stented valve	Ovine dermal fibroblasts	<i>In vitro</i> bioreactor conditioning	Minimal regurgitant fractions and transvalvular pressure gradients; Large effective orifice areas; Maintained mechanical properties after fatigue testing; No observable macroscopic deterioration	Syedain et al. (2013)
2013	Molding, decellularization, <i>in situ</i> engineering	Polyglycolic-acid coated with poly-4-hydroxybutyrate (P4HB)	Initially seeded with vascular cells then decellularized	Self-expandable stented valve	Human vascular-derived fibroblast	<i>In vitro</i> bioreactor conditioning (4 weeks); <i>in vivo</i> implantation into non-human primate pulmonary position (8 weeks)	Mild and moderate valve insufficiency observed; Mobile and thin leaflets; Rapid recellularization (VIC and VEC) and ECM remodeling (collagen, GAG)	Weber et al. (2013)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2014	Decellularization	Porcine aortic valves	Decellularized valve treated with PEG; fixed in glutaraldehyde and detoxified	Stented valve	N/A	<i>In vitro</i> bioreactor testing (100 days); <i>in vivo</i> implantation in sheep (mitral valve position, 18 months)	Functional valve after 18 months; No observed immune reaction or calcification	Lim et al. (2014)
2014	Decellularization	Ovine aortic valves	Decellularized valve coated with fibronectin and stromal cell-derived factor-1 α	Whole valve	N/A	Implanted in sheep pulmonary position	Coated valves had no observable calcification; Lack of immune response; Preservation of collagen network organization and density; Improved re-endothelialization	Flameng et al. (2014)
2014	Electrospinning	Poly(ethylene glycol) dimethacrylate-poly(lactic acid)		Whole valve	Porcine VICs and VECs	<i>In vitro</i> characterization	Similar mechanical properties to native valve (fiber diameter, pore size, tensile strength); Functional within bioreactor environment; Maintenance of VIC and VEC phenotype	Hinderer et al. (2014)
2014	Molding, decellularization, <i>in situ</i> engineering	Polyglycolic-acid coated with poly-4-hydroxybutyrate (P4HB)	Initially seeded with vascular cells then decellularized	Self-expandable stented valve	Human/ovine vascular cells	<i>In vitro</i> bioreactor conditioning (4 weeks); <i>in vivo</i> implantation into ovine pulmonary position (24 weeks)	Mild to moderate regurgitation; Cellular repopulation and matrix remodeling	Driessen-Mol et al. (2014)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2014	Molding	Self-assembled fibroblast sheets (8 layers)		Whole valve	Human dermal fibroblasts	<i>In vitro</i> bioreactor conditioning	Uniform distribution of cells and collagen matrix; Lack of trilayer ECM composition; Functional valve in bioreactor	Tremblay et al. (2014)
2014	Molding	Self-assembled fibroblast sheets (9 layers)	Leaflets formed by self-assembled sheets cut and sutured onto stent	Stented valve	Human dermal fibroblasts	Bioreactor conditioning	ECM composed mainly of collagen; UTS matched other TEHV but had lower elastic modulus; Functional within bioreactor	Dube et al. (2014)
2014	Electrospinning	Poly(caprolactone) and poly(ethylene glycol) diacrylate	Incorporated PCL fibers into PEG hydrogels and seeded cells on top	Tissue scaffold	Porcine aortic VICs	<i>In vitro</i> characterization of scaffold	Achieved anisotropic mechanical properties; Cells aligned in direction of underlying fibers	Tseng et al. (2014)
2014	Electrospinning	Poly(glycerol sebacate)-poly(s-caprolactone) and methacrylated hyaluronic acid/methacrylated gelatin	Immersed PGS/PCL fibers into Me-HA/Me-Gel solution and cross-linked; cells seeded on PGS/PCL and encapsulated in Me-HA/Me-Gel	Tissue scaffold	Ovine mitral VICs	<i>In vitro</i> characterization of scaffold (21 days static)	Enhanced cell spreading and metabolic activity within composites	Eslami et al. (2014)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2015	<i>In vivo</i> engineering	Silicone rod, polyurethane scaffold		Self-expandable stented valve	N/A	<i>In vivo</i> synthesis in goat; <i>In vivo</i> implantation aortic position in goat	Functional valve within goat	Kishimoto et al. (2015)
2016	Decellularization	Human donor aortic and pulmonary valves		Whole valve	N/A	<i>In vitro</i> characterization; mouse subcutaneous implant model (12 weeks)	Retention of collagen IV and vWF but loss in fibronectin laminin, and chondroitin sulfate; Reduced GAG content; Compatible in mouse model; No decrease in mechanical properties	Vafaei et al. (2016)
2016	Molding, <i>in vivo</i> engineering	Acrylic mold with self-expanding stent		Tubular valve	N/A	<i>In vivo</i> tissue regeneration, <i>in vitro</i> characterization	Enhanced leaflet kinematics compared to old Lower regurgitant fraction	Sumikura et al. (2016)
2017	Electrospinning, decellularization	PCL, chitosan, bovine pericardium	Electrospinning of PCL-chitosan combined with decellularized bovine pericardium	Valve leaflet	Human aortic VICs	<i>In vitro</i> characterization of scaffold (mechanical testing, biocompatibility)	Mechanically stronger than native valves; Cells proliferated and deposited dense ECM	Jahnavi et al. (2017)

(Continued)

Table 10.1 (Continued) Summary of Selected Tissue Engineered Heart Valve Studies

Year	Technique	Material	Additional Details	Type of Construct	Cell Seeding	Study Type	Results	References
2017	Mechanical	Nitinol	Leaflets formed via nitinol scaffold attached to a stent	Valve leaflet	Smooth muscle cells, fibroblast/myofibroblast	<i>In vitro</i> bioreactor characterization; computational modeling	Enhanced cell growth and collagen and elastin deposition; Proper opening and closing of leaflets	Alavi et al. (2017)
2017	Electrospinning, <i>in situ</i> engineering	Bis-urea-modified polycarbonate (PC-BU)		Stented tubular valve	N/A	<i>In vitro</i> characterization; <i>in situ</i> remodeling in pulmonary position in lamb (12 months)	Complete cellular infiltration; No calcification or thrombosis; ECM deposition (collagen, GAG, elastin); Anisotropic mechanical properties	Kuin et al. (2017)
2017	Decellularization, molding	Decellularized porcine small intestinal submucosa		Tubular valve	N/A	<i>In vivo</i> implantation in pigs (6 months)	Decreased leaflet area and height; Increased regurgitation volume and fraction	Ropcke et al. (2017)

Specifically for heart valves, the most common method used includes chemical treatment (e.g. SDS, Triton X-100, or the combination of the two) in conjunction with nucleases (Liao et al. 2008; VeDepo et al. 2017; Abdolghafoorian et al. 2016).

These valves offer the quickest route to the clinic. Researchers have already performed both animal (Akhyari et al. 2010; Paniagua Gutierrez et al. 2015; Takagi et al. 2006) and human (Neumann et al. 2014) studies. To date, there are a few decellularized tissue-engineered heart valves in the market. In the United States, the CryoValve SynerGraft® family (aortic valve and SG pulmonary valve) is the only acellular valve approved. However, in Europe, there are two companies offering decellularized valves: (1) AutoTissue GmbH Matrix P plus N™ and Corlife Arise AV® and (2) Espoir PV®. The CryoValve and Corlife valves are allografts, whereas AutoTissue valves are xenografts, derived from porcine tissue.

Decellularized valves have varying degrees of success initially, but recent clinical data show promising results. Initial studies from the SynerGraft® were fatal for three of four children due to inflammatory responses (Simon et al. 2003). Short-term follow-up with the valve used in the Ross procedure for adults proved more favorable, showing no deaths, reoperations, or valve deterioration (Brown et al. 2011). More recently, an intermediate- and long-term follow-up showed the SynerGraft had lower stenosis, conduit dysfunction, and pulmonary insufficiency when compared to standard allografts, potentially due to the decreased immunogenicity (Bibevski et al. 2017). AutoTissue's Matrix P™ acellular valves showed variable results as well. While the valve performed similar to native valves, a third of the patients needed additional surgeries (Konertz et al. 2005). In other studies where patients were predominately neonatal, infant, or children, about a third of the cohort experienced conduit failure and conduit dysfunction (Perri et al. 2012). Failure of these grafts reached above 50% in other cases, which were attributed to inflammation and fibrosis (Voges et al. 2013). The Corlife valves showed favorable results in mid-term results in the pulmonary position with higher freedom from explantation and intervention when compared to conventional allografts and xenografts (Sarikouch et al. 2016).

Although some of the decellularized valves show promising results, especially in the more recent studies, there are still many research questions to be addressed. One critical weakness of decellularized valves is the seemingly unpredictable graft failure, which could be caused by immunogenicity or incomplete recellularization, so further work in elucidating mechanisms of failure and prevention is crucial (Cicha et al. 2011). Other advances in the field include analyzing the effects of different decellularization components on maintaining tissue architecture and immunogenicity and recellularization potential, although some researchers question whether complete recellularization *in vitro* is needed (Vafaei et al. 2016; Dohmen et al. 2006b; Roosens et al. 2016). In addition, many of these

valves are allogenic, which is not the most viable options, especially for pediatric patients due to limited availability. Further research is needed to understand and enhance cellular infiltration, remodeling, and reendothelialization while decreasing immunogenicity and calcification.

10.5.2 Polymeric valve molding

Many tissue-engineered heart valves predominantly came from molding of bio-materials to form a simple valve geometry or fabricate leaflets and suture them onto a stent, similar to what current bioprosthetics look like (Figure 10.3a) (Sewell-Loftin et al. 2011; Claiborne et al. 2012). Initial studies from the late 1900s and early 2000s generally consisted of using synthetic, biocompatible, and polymeric

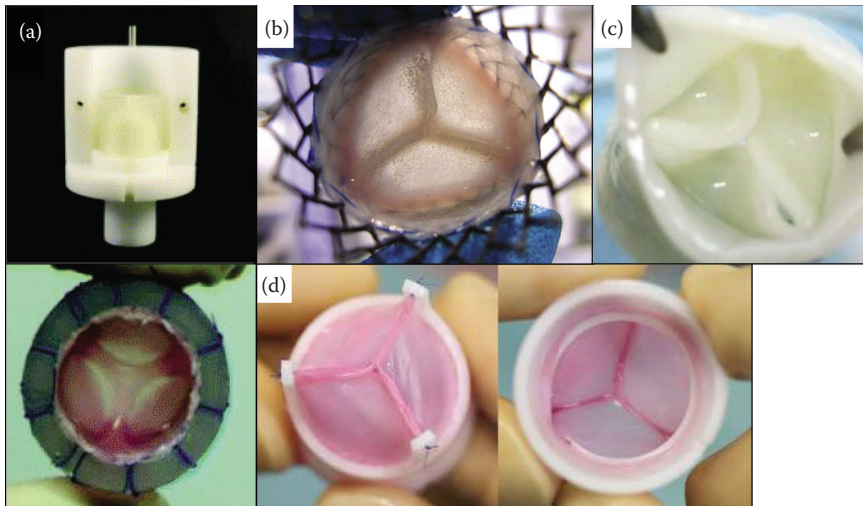


Figure 10.3 Molding techniques for heart valve fabrication. (a) Assembled injection mold for elastin-like recombinamer (ELR) and fibrin hybrid gels. Fabricated valves were supported with a silicone ring. (From Torre, I.G.de. et al., *Biomater. Sci.*, doi:10.1039/C6BM00300A, 2016. With permission.) (b–d) Tubular molds. (b) Decellularized nonwoven polyglycolic-acid meshes (PGA) coated with poly-4-hydroxybutyrate (P4HB) were integrated into a self-expanding nitinol stent. (From Weber, B. et al., *Biomaterials*, 34, 7269–7280, 2013. With permission.) (c) Two concentric engineered fibrin tubes are attached together via degradable sutures to form the belly and commissural regions. (From Reimer, J. M. et al., *Biomaterials*, 62, 88–94, 2015. With permission.) (d) Tubular valve fabricated from self-assembled cell sheets formed on a series of cylindrical molds was sutured on a custom valve holder such that the valve folds inward to form a fully coapt trileaflet valve. (From Picard-Deland, M. et al., *Ann. Biomed. Eng.*, doi:10.1007/s10439-016-1708-1, 2016. With permission.)

blends of material, including silicone, polytetrafluoroethylene (PTFE), polyurethanes and blends, polyglycolic acid (PGA), polylactic acid (PLA), polyhydroxyalkanoate (PHA), and poly-4-hydroxybutyrate (P4HB) (Breuer et al. 1996; Ralf Sodian et al. 2000; R. Sodian, Hoerstrup, Sperling, Martin, et al. 2000; Ghanbari et al. 2009). Synthetic polymers are an attractive option for engineering valves because of the tunability of mechanical and chemical properties to match native valve properties (Pérez et al. 2013). Researchers have the flexibility to combine different polymers with unique properties to tune leaflet flexibility, cell adhesion, anisotropic mechanical properties, and degradation rates (Ralf Sodian et al. 2000; R. Sodian, Hoerstrup, Sperling, Daebritz, et al. 2000). Using the standard TE paradigm, these molded or sutured scaffolds are usually seeded with cells for remodeling and conditioned in a pulsatile or cyclic bioreactor for further *in vitro* development before being implanted into an animal model (Sodian et al. 1999; R. Sodian, Hoerstrup, Sperling, Daebritz, et al. 2000). In addition, these valves are able to be molded and attached to self-expanding stents for TAVI/TAVR procedures, which a few groups have performed in ovine and in primates (Figure 10.3b) (Weber et al. 2013; Driessen-Mol et al. 2014). Early iterations of these valves, while they can be molded into leaflets or whole valves on a stent, possessed several major problems, including short durability, leaflet stenosis, thrombosis, and calcification—all elements of valvular pathology (Ghanbari et al. 2009). Scaffold degradation rate can also be a challenge. If the scaffold degrades faster than ECM production, the valve may fail prematurely due to lack of mechanical integrity, resulting in valvular incompetency (Claiborne et al. 2012; Ghanbari et al. 2009). Conversely, if the degradation rate is too slow or incomplete, chronic inflammation may occur, which can lead to fibrosis, stenosis, and calcification.

In addition to synthetic polymers, some groups have used naturally derived material for molding. Collagen is a major component in valves, and purified collagen hydrogels can be synthesized, making this material one popular choice for researchers to use (Taylor et al. 2002). Human VIC encapsulated in collagen matrices maintained native phenotypic markers, enhanced cellular proliferation, and facilitated cells for tissue remodeling (Taylor et al. 2006; Tedder et al. 2009). Other groups have shown preferential cell and matrix fiber alignment within collagen hydrogels (Butcher and Nerem 2006; Neidert and Tranquillo 2006). Unfortunately, collagen-based scaffolds have mostly been confined to *in vitro* testing (Tedder et al. 2011), potentially owing to its compaction potential and restricting its utility for heart valve engineering (Feng et al. 2014; Farrar et al. 2016).

Fibrin perhaps has been the more popular material for TEHV. Fibrinogen can be isolated from a patient's own blood, so once combined with thrombin, an autologous fibrin scaffold can be produced, which has immediate clinical applications (Ye et al. 2000). In addition to the possibility of making autologous scaffolds, fibrin has been a popular choice due to its biocompatibility within the host and the

ability for cells to infiltrate the scaffold (Ahmed et al. 2008; Flanagan et al. 2009). Fibrin can also promote collagen production and enhance GAG retention within the developing ECM (Alfonso et al. 2013). Researchers have seeded vascular cells onto molded fibrin scaffolds and implanted the TEHV into sheep (Flanagan et al. 2007, 2009). The explants showed qualitatively similar matrix organization when compared to native valves. However, fibrin and other biological protein-based TEHV are susceptible to cell-mediated contractions, which could be due to the stress differential between the stress generated by the leaflets (i.e. cells) and the stress on the leaflets (i.e. diastolic pressure), leading to leaflet retraction or weakened scaffold (van Loosdregt et al. 2014). To overcome this limitation, some groups have combined naturally derived polymers with other materials, synthetic or naturally derived. One group has developed a scaffold that contained a mixture of fibrin-coated collagen–glycosaminoglycans sheets that yielded stronger mechanical properties than fibrin scaffolds and showed no cell-mediated reduction in scaffold size after culturing *in vitro* (Brougham et al. 2015). Others have fabricated elastin sheets on top of hydrogels containing cross-linked hyaluronan, but these have yet to be used *in vivo* (Ramamurthi and Vesely 2005).

One currently popular method of molding is forming a tubular valve. Tranquillo and his colleagues developed a tubular mold around which the TEHV is formed. The fabricated fibrin-based tubular valve is attached onto a valve frame with three prongs, and the back pressure causes the excess tissue to coapt, forming the leaflets (Syedain et al. 2013). In another instance, his group developed a stentless tube-in-tube model where the inner tube (the leaflets) was sutured on to the outer tube (valve root) (Figure 10.3c) (Reimer et al. 2015). Both types of valves performed well under pulmonary conditions *in vitro* without observable damage after at least 1 million cycles of fatigue testing and were comparable to either a native porcine valve or a commercial bioprosthetic. The stented version was implanted into sheep up to 6 months and showed repopulation of both interstitial-like and endothelial cells, new ECM deposition, and retention of mechanical properties (Syedain et al. 2015). The tubular version targeted for pediatric patients showed functionality up to 8 weeks in a growing sheep model (Reimer et al. 2017). Although the valve was repopulated with cells that deposited elastin and collagen IV, the valve had an increased insufficiency index after the sutures degraded, potentially due to inadequate fusion of the commissures. The Auger group has used self-assembled cell sheets as another material to produce a stented tubular valve design withstanding up to 70 mmHg peak transvalvular pressure, which has shown great potential for TEHV applications (Figure 10.3d) (Dubé et al. 2014; Picard-Deland et al. 2016).

While many molded designs have shown great potential *in vitro* and *in vivo*, there are still challenges to be addressed. One major drawback with molding is the eventual retraction of the leaflets, leading to valvular insufficiency (Flanagan et al. 2009; Gottlieb et al. 2010). There are recent advances in techniques to

circumvent this problem. Sanders et al. developed a new mold insert to maintain TEHV geometry by reducing leaflet tissue compression in the radial direction (Sanders et al. 2016). *In vitro* results indicated decreased radial compression, and the authors expect less leaflet retraction on implantation. Others have overcompensated the design such that on scaffold contraction *in vitro*, the sizing of the TEHV would be as specified (Picard-Deland et al. 2016). Many of these designs lack the anatomical shape of the aortic root because of the complexity of the geometry, leaving out important features, including the sinuses. Although there is potential to incorporate multiple materials to fabricate a layered TEHV mimicking the native trilayer ECM of the leaflet, most of the currently molded TEHV are heterogeneous in nature, using the same material for both the leaflets and the root. The lack of material heterogeneity can make it difficult to match native anisotropic biomechanical properties of both tissue types.

10.5.3 Electrospun nanofibrous scaffolds

Fully artificial scaffolds, including porous, fibrous, and hydrogel scaffold, have been fabricated for TE heart valve by employing synthetic, natural polymers, or polymer blends over the past two decades (Jana et al. 2014). In contrast to porous or hydrogel-based heart valve replacement, fibrous constructs preserve multiscale features of natural valves, which possess the ability to encourage endogenous remodeling after somatic transplantation. Currently, nanofiber-based scaffolds have been demonstrated to give the most promising outlook in respect to TEHV applications (Hasan et al. 2016; Namdari and Eatemadi 2016). Nanofibers possess long continuous structures with diameters on the order of native ECM fibers (50–1000 nm), which resemble the inherent cellular environment well. Moreover, fibrous structures facilitate to handle tensile loads and maintain relatively low bending rigidity. A wide array of mechanical behaviors of scaffolds can be produced and adjusted by controlling the distribution of fibers during manufacturing. Finally, scaffolds composed predominantly with nanofibers provide high porosity and high surface area to volume ratios. These characteristics encourage cell adhesion and serve as a great framework for cell proliferation and differentiation.

Although there are many other methods of manufacturing nanofibers, such as template synthesis, self-assembly, and phase separation in the nanotechnology field, electrospinning is recognized as the simplest method to produce nanofibers in terms of its versatility, flexibility, and ease of fiber production (Wu et al. 2016b). A commonly lab-used electrospinning setup only needs a blunt tip needle, a syringe, an injection pump, a high-voltage power supply, and a nanofiber collector. Electrospun nanofibers have been extensively investigated for applications in TEHV (Wu et al. 2014).

A broad range of polymers have been electrospun and evaluated for their ability to support the regeneration of heart valve leaflets. Chitosan is known to be a

biodegradable, biocompatible, and bioactive natural polymer, and studies have demonstrated the efficacy of chitosan nanofibers as scaffolds for valve leaflet substitutes *in vitro* (Albanna et al. 2012). However, it is difficult to produce chitosan nanofibers due to its poor solubility in widely used organic solvents and its ionic nature in dissolved form. Hence, chitosan has been physically blended with other natural and synthetic polymers such as collagen, poly(ϵ -caprolactone) (PCL), and so on (Fu et al. 2017; Gallyamov et al. 2014). Chitosan–collagen nanofibers have been reported to have good cytocompatibility and also enhance tissue regeneration *in vitro*. (PGA, poly-L-lactide (PLLA), poly(ester urethane) urea (PEUU), poly(glycerol sebacate) (PGS), and their blends are the known synthetic polymers that have been employed to produce fibrous TEHV's using electrospinning (Amoroso et al. 2012; Lueders et al. 2014; Fan et al. 2013; Masoumi et al. 2013).

Kluin et al. depicted a breakthrough to use electrospinning-based scaffold for TEHV application (Figure 10.4) (Kluin et al. 2017). They employed a novel supra-molecular elastomer (polycarbonate bisurea) to fabricate a nanofibrous valvular scaffold that enables to sustained functionality up to 12 months as pulmonary

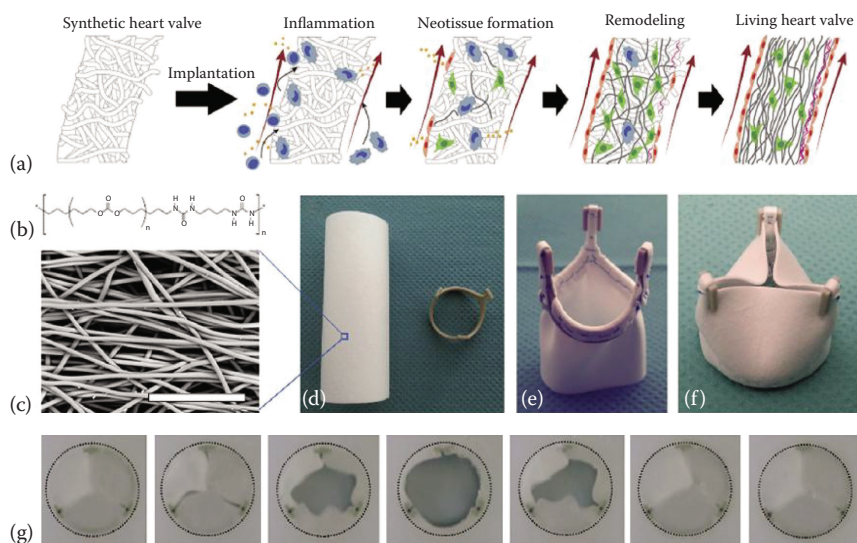


Figure 10.4 Fabrication and *in vitro* functionality of the polycarbonate bisurea (PC-BU) valve. (a) Schematic representation of the hypothesized phased process of heart valve regeneration. (b) The strictly segmented, sequence-controlled molecular structure of PC-BU. (c) Scanning electron microscopy image of the fibrous microstructure of the valve. Scale bar: 50 mm. (d–f) The valve is composed of an electrospun PC-BU tube sutured onto a reinforcement crown. (g) Movie stills of an *in vitro* valve functionality test demonstrating good closure and opening behavior. (From Kluin, J. et al., *Biomaterials*, 125, 101–117, 2017. With permission.)

valve in sheep, whereas the implant was gradually replaced by a layered collagen and elastic matrix in pace with cell-driven polymer resorption. However, typical electrospinning processes create random aligned nanofiber mats that poorly mimic the anisotropic feature of native heart valve tissues. To remedy this, several modified electrospinning techniques have been developed to manipulate the nanofiber deposition during manufacturing. Some groups employed high-speed rotating cylinder to fabricate uniaxially aligned nanofiber meshes, to mimic the intrinsic anisotropy of native heart valve leaflets (Eckert et al. 2011; Gaharwar et al. 2014). Masoumi et al. utilized directional electrospinning to collect aligned PGS:PCL fibrous scaffold, which resemble native heart valve leaflet ECM networks. They further changed the ratio of PGS:PCL to tune the anisotropic mechanical characteristics of fabricated scaffolds to mimic the native heart valve's mechanical properties (Masoumi et al. 2014b). Jana et al. electrospun a unique morphologically biomimicked, pliable, but stand-alone substrate with circumferentially oriented nanofibers on a novel collector, which resembled the fibrosa layer of a cardiac aortic valve. Hobson et al. developed a new method to produce heart valve-like curvilinear fiber alignment in electrospun scaffolds by varying the geometry of the collecting mandrel, which would homogenize the strain field and reduce stress concentrations at the commissure (Hobson et al. 2015).

The native valve leaflet ECM exhibits complex 3D structure and is organized into three layers: (1) ventricularis, mainly containing radially aligned elastin fibers; (2) spongiosa, with randomly distributed glycosaminoglycans (GAGs) and proteoglycans; and (3) fibrosa, consisting of circumferentially aligned collagen fibers. However, electrospun nanofibrous scaffolds usually possess 2D construction, which is difficult to mimic the 3D complex structure of native ECM of valve leaflets. Therefore, electrospinning may be combined with other scaffold fabrication technologies to compensate for this potential limitation. Jahn timer et al. reported a biohybrid scaffold with noncross-linked decellularized bovine pericardium extracellular matrix (DBPECM) coated with a layer of electrospun polycaprolactone–chitosan nanofibers, which exhibited most of the physical, biochemical, and functional properties of the native valve (Jahn timer et al. 2015). Masoumi et al. fabricated a trilayered scaffold by combining electrospinning and microfabrication techniques, which opened and closed properly in an *ex vivo* model of porcine heart valve leaflet tissue (Masoumi et al. 2014a). Another approach for enhancing electrospun nanofibrous scaffold replacement is embedding electrospun fibres into bioactive hydrogels (Tseng et al. 2014; Eslami et al. 2014). These fiber-enhanced hydrogel composite scaffolds are potentially implemented in preclinical models due to their capability of mimicking the physical and biological features of heart valve tissue.

Although electrospinning can fabricate nanofibrous scaffolds with random or aligned fiber architecture that possess ECM-like structures, one of the unmet challenges of this sheet-like nonwoven morphology is their very small pore size

(less than 5 μm), which prevents cells from penetrating into the scaffolds and are not beneficial for the transportation of oxygen and nutrients throughout the implant site and removal of metabolic waste during tissue regeneration. But even worse, electrospun nanofibrous scaffolds usually possess poor suture-retention strength for the surgical reconstruction and are insufficiently strong to provide mechanical support to primary repairs. Thus, while electrospinning permits fabrication of nanofibrous matrices that resemble the scale and architecture of the native ECM, achieving high cellular density and infiltration and excellent mechanical properties remains challenging.

To overcome the aforementioned challenges, Wu et al. developed a living nano–micro fibrous woven fabric/hydrogel composite scaffolds for TEHV by employing nanofiber yarn-processing technique, and textile technique combined with bioactive hydrogel formation ([Figure 10.5](#)) (Wu et al. 2017). First, they developed and patented a novel electrospinning approach for the continuous fabrication of uniaxially aligned nanofiber yarns (Wu and Qin 2013). The nanofiber yarns have been demonstrated to fabricate from various polymers, such as polyacrylonitrile (PAN), polycaprolactone (PCL), and polyurethane (PU), and so on (Wu et al. 2016a). Moreover, the nanofiber yarn exhibited high tensile strength and Young's modulus and can be easily processed into near limitless heterogeneous and anisotropic scaffolds by textile techniques. Then, PAN nanofiber yarns were used as the weft yarns to pass through PAN microfiber yarns (the wrap yarns) to form the mechanically stable weaving fabrics by textile-weaving approach. The microstructure of the woven scaffold with controlled strength, porosity, morphology, and geometry can be adjusted to meet the mechanical properties of the native valve leaflets, which are beneficial for the valve cells infiltration and can enhance the transport of nutrients and oxygen to cells and remove the metabolic waste in time. Following the textile-weaving technique, the living cell-laden methacrylated hyaluronic acid (Me–HA)/methacrylate gelatin (Me–Gel) bioactive hydrogel precursor was added to the PAN nano–microfibrous woven fabrics and photocross-linked by UV light to form the composite scaffolds. This strategy combines the properties of ECM-mimicking hydrogel and anisotropic woven fibrous biomesh to provide both elasticity and anisotropy. The composite constructs can mimic the structural and mechanical properties of native aortic valve leaflets while simultaneously supporting the cell growth and tissue formation with controlled microarchitecture. Moreover, the composite scaffolds support the growth of human aortic valve interstitial cells (HAVIC), balance the remodeling of heart valve ECM against shrinkage, and maintain better physiological fibroblastic phenotype in both normal and diseased HAVIC over single fabric or hydrogel materials. The nano–micro fibrous woven fabric-enhanced hydrogel composite scaffolds represent a promising TE strategy to treat heart valve injury.

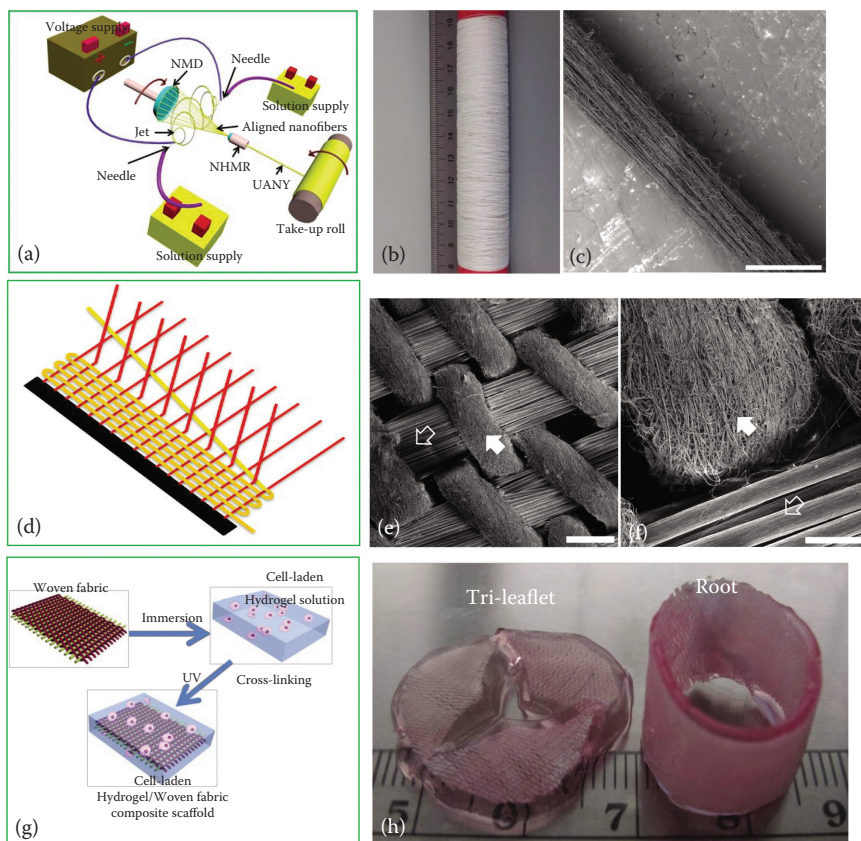


Figure 10.5 Living cell-laden nano-micro fibrous woven fabric/hydrogel composite scaffolds for heart valve engineering application. (a) Schematic illustration of the novel electrospinning setup and its use to continuously fabricate nanofiber yarns. (b) Photograph of a PAN UANY package produced in about 6 hours using the novel electrospinning setup. (c) FESEM images of PAN nanofiber yarns. Scale bars = 200 μm . (d) Schematic of the weaving process that requires passing weft yarns through warp yarns. (e–f) FESEM images of the obtained PAN nano-micro fibrous woven fabric. Scale bars: 200 μm for (e) and 50 μm for (f), respectively. The solid arrows and hollow arrows indicate PAN nanofiber yarns and PAN microfiber yarns, respectively. (g) Schematics of the design and fabrication of composite scaffold. (h) Photograph of the trileaflet and aortic root with inner diameter of 15 mm. (Reprinted with permission from Wu, S. et al., *ACS Appl. Mater. Interfaces*, 8, 16950–16960, 2016a. Copyright 2016 American Chemical Society.)

10.5.4 *In vivo/in situ* engineering of heart valves

Much of the classical TEHV research attempts to reduce immunogenicity of the fabricated scaffolds by using autologous cell sources or highly biocompatible material. Some groups, however, harness the foreign body response to regenerate tissue, effectively using the body as a bioreactor. There have been attempts of *in situ* TEHV that was combined with more traditional methods, including decellularized xenografts (Zafar et al. 2015) and allografts (Theodoridis et al. 2015; Tudorache et al. 2016) and *de novo* engineered valves (Weber et al. 2013, 2013; Syedain et al. 2015). A couple of groups have instead generated a mold to be implanted into animals such that a layer of tissue is generated on the mold, which is then used to form a TEHV (Kishimoto et al. 2015; Funayama et al. 2015; Sumikura et al. 2014). The latest development of this *in vivo* generated biovalve used a new self-expanding and stent-mounted mold that consisted of three hemispheres to produce round-shaped leaflets to enhance leaflet coaptation (Sumikura et al. 2016). After implantation in goat pouches for two months, the tissue was generated on the mold, which was folded inward to form three commissural parts with three leaflets. The valve behaved similar to commercially available biological valves except with higher regurgitation. In addition, altering the diameter of the valve (19–25 mm) altered pressure difference, effective orifice area (EOA), and regurgitant fraction, indicating that this technique may work for a limited range of valve diameters.

This technique has some advantages over traditional methods by reducing the complexity of the fabrication system by eliminating donor tissues and/or cells. If the scaffold to be implanted is synthetic, there can also be reduced cost and regulatory complexity while maintaining high control of the scaffold properties (Kluin et al. 2017). It is autologous by nature and may eliminate any acute immunogenic response on transferring to the heart. However, similar to many other fabrication methods, it currently lacks the ability to recapitulate the whole valve geometry, including the sinuses. The mechanical properties can exceed native properties, which could potentially illicit a pathological response for the long term, leading to calcification.

10.6 3D printing for heart valves

3D printing, which is often referred to as additive manufacturing, rapid prototyping, or solid free-form fabrication, is an advanced and highly specific manufacturing technique to fabricate complex geometries through computational models, generally through detailed computer-aided designs (CAD) and automated processes to create 3D objects (Murphy and Atala 2014; Duan et al. 2014). When compared to other biofabrication techniques, 3D bioprinting enables the production of 3D constructs with precise controlled architecture, allows for the use of multiple cell types within the same construct, and creates more physiologically

relevant microenvironments (Murphy and Atala 2014). Ultimately, this can lead to recreation of complex, heterogeneous, and anisotropic tissues accurately.

Similar to 3D printing of other complex tissues, the general process of printing a heart valve follows a typical format. First, a noninvasive 3D scan of the valve is performed via CAT or MRI to acquire a patient-specific model (Muraru et al. 2016). The model is then processed and segmented to identify different components of the tissue for printing. After processing and converting to a printer-readable file (e.g. STL), the model can be printed using a blend of user-defined bioinks, which are biomaterials that consist of biodegradable polymers (natural or synthetic), hydrogels, or spheroids—all of which may include encapsulated cells.

10.6.1 Clinical 3D printing of heart valves

At the onset of the technology, 3D printing had an immediate impact in the clinic. Cardiovascular surgeons have already utilized 3D-printing technology to generate patient-specific models prior to surgical interventions to visualize the procedure and plan accordingly (Gerstle et al. 2014; Sodian et al. 2008). These reconstructive models have been used for both congenital defects and aortic valve replacements (Sodian et al. 2007; Ralf Sodian, Schmauss, et al. 2008; Ralf Sodian, Weber, et al. 2008; Ralf Sodian et al. 2009; Schmauss et al. 2012). While the structures were nonliving and made of thermoplastics, the early studies paved way for tissue engineers to modify the existing techniques for bioprinting (Peltola et al. 2008).

10.6.2 Bioprinting TEHV

Among the several types of 3D-bioprinting strategies—including inkjet, laser-assisted, and extrusion—fabrication of TEHV has mainly been based on extrusion-based bioprinting (EBB). The main advantages of using EBB for TEHV are the ability to balance out the printing time, shape fidelity, and spatial and temporal distribution of cell types (VIC and SMC) within mechanically heterogeneous materials corresponding to the leaflets and root, respectively.

Several different valve models and designs have been used for 3D bioprinting. As an initial proof of concept, Duan et al. designed a flat valve model using a bio-ink blend of methacrylated gelatin and methacrylated hyaluronic acid with human aortic VIC encapsulated (Figure 10.6a–c) (Duan et al. 2014). The polymer ratio and concentration were optimized to control the hydrogel viscosity and construct stiffness. The encapsulated cells had high viability and matrix remodeling ability with sulfated GAG deposition (Figure 10.6c). One major concern about this testing model is that the model does not contain any sinus or commissure, which is needed to relieve abnormal stress and prevent blood back flow. Hockaday et al. had an improved design with an axially symmetric shape and used poly(ethylene glycol)

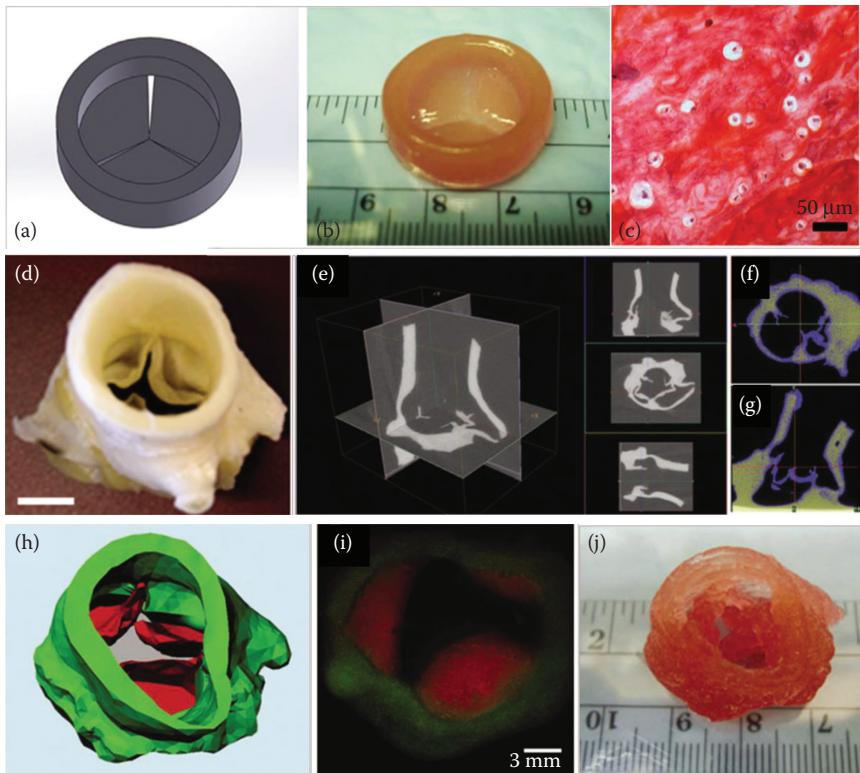


Figure 10.6 3D bioprinting of heart valves. (a–c) Flat valve without sinus and commissural structure. (From Duan, B. et al., *Acta Biomaterialia*, 10, 1836–1846, 2014. With permission.) (a) Flat valve CAD model. (b) Bioprinted flat valve using methacrylated hyaluronic acid and methacrylated gelatin. (c) Safranin-O staining of the printed TEHV. (d) Preserved porcine aortic valve. (e–g) MicroCT scan slices and their reconstruction into a valve model for 3D printing. (From Hockaday, L.A. et al., *3D Printing*, 1, 122–136, 2014. With permission.) (h–i) Anatomically accurate bioprinted heart valves. (From Hockaday, L.A. et al., *Biofabrication*, 4, 35005, 2012. With permission.) (h) The valve scans were viewed, thresholded, and segmented into separate STL files for the leaflet (red) and the root (green). (i) Fluorescent image of first printed two layers with cell tracker stained cells (SMC in green and VIC in red). (j) Bioprinted hydrogel valve using PEGDA.

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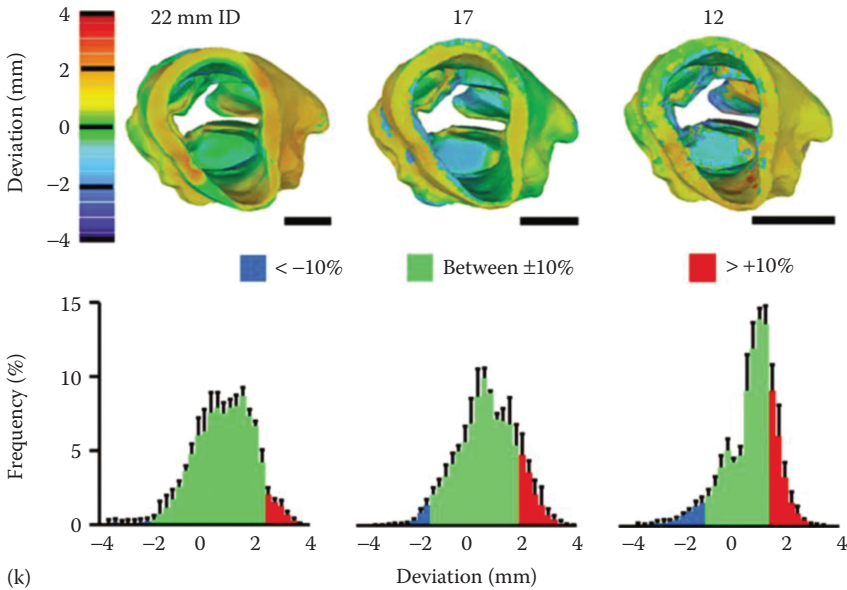


Figure 10.6 (Continued) 3D bioprinting of heart valves. (k) Heat maps and average frequency histograms representing surface deviations between printed porcine scaffolds with different sizes representing pediatric to adult versus 3D model geometries.

diacrylate (PEGDA) to print valve conduits with biomechanical heterogeneity (Hockaday et al. 2012). In this design, the leaflets were printed using PEGDA with high molecular weight (8000 MW) and root was printed using low molecular weight (700 MW) PEGDA. The printed valve thus had more flexible valve leaflets and relatively rigid valve root. The same group also used microCT to scan a freshly obtained porcine aortic valve (Figure 10.6d) and generated an aortic valve geometric model (Figure 10.6e–f) (Hockaday et al. 2014). Hydrogel valves have been printed to span a range of clinically relevant sizes from a 6-month infant aortic valve (as small as 12 mm inner diameter) to an adult size (around 22 mm inner diameter) (Hockaday et al. 2012). The shape fidelity of the printed valves was measured and characterized using surface deviation analysis by comparing microCT imaging with the design (Figure 10.6h). The accuracy decreased with decreasing the valve size, but the overall shape fidelity was higher than 70% even for the printed pediatric valve. This model maintained the normal anatomical characteristics of native valves, such as the ostium and sinus. 3D bioprinting also enables multiple cells and material printing to generate heterogeneous valve conduits. As shown in Figure 10.6i, the anatomical-shaped valve model was separated, based on the tissue density difference, into valve leaflet and valve root. Then, the valve root, made of SMC-laden hydrogel, was deposited first and subsequently, the leaflet

region of the layer, made of VIC-laden hydrogel, was extruded along its print paths (Figure 10.6j) (Duan et al. 2013). These exhibited geometry comparable to the original image of the derived valve (Figure 10.6k) (Bin Duan et al. 2013).

While several of these studies show advances in 3D bioprinting for TEHV, there remains strategy-dependent challenges that need to be addressed to further improve the process of fabricating a valve. Neither inkjet nor laser-assisted bioprinting strategies, despite having higher print resolution than extrusion-based bioprinting, have been used to fabricate heart valves, potentially due to the long print time. In an extrusion-based system, it is difficult to print the trilayer structure of the valve, given the micron resolution needed. However, this may not be an issue if encapsulated cells *in vitro* or infiltrated cells *in vivo* remodel the tissue into the native trilayer structure (Kluin et al. 2017; Reimer et al. 2017; Driessen-Mol et al. 2014). Many extrusion-based printers with UV cross-linking capabilities will generally have the UV source path over the printed layer. Over a course of a large tissue print, such as a heart valve, there could be potential UV bleed through to the bottom layers, thus creating a gradient of cross-linked material from bottom (most cross-linked) to top (least cross-linked). This potential problem can be circumvented via modeling of UV light penetration through the material and optimizing the time at which the UV light is exposing the material. Alternatively, a strong, focused point UV source can be aimed directly below the extrusion tip to immediately cross-link the extruded biomaterial. The other two bioprinting strategies—(1) inkjet and (2) laser-assisted—have yet to be used for fabricating heart valves, potentially largely due to the long print time.

10.7 Conclusion and future remarks

Advances in 3D bioprinting of heart valves have shown great potential toward treating end-stage valve disease. Bioprinting possesses the ability to fabricate complex, patient-specific geometries with macro- and microscale architecture in a heterogeneous mechanical environment that supports multiple cell types. Although there are a few types of techniques for bioprinting, extrusion-based bioprinting remains the only strategy used to form heart valves, which ranges from a simple, homogeneous structure to a more complex, heterogeneous scaffold. Despite advances in technologies for TEHV bioprinting, there are still some challenges that need to be addressed before moving toward the clinic. In particular, other types of biomaterials should be explored to enhance cell integration or differentiation to drive tissue remodeling to the native trilayer state while maintaining biocompatibility and printability. In addition, improvements on methods to enhance spatial resolution at the microscale would be useful to recapitulate minute structures and increase shape fidelity. Finally, fabrication reproducibility and consistency must be

achieved not only to produce accurate scaffolds but to adhere to future regulations (e.g. cGMP). Addressing these issues may further help researchers move toward *in vivo* studies with the eventual goal of moving toward the clinic.

References

- Abdolghafoorian, H., P. Farnia, S. N. Raheleh Sadat, A. Bahrami, A. Dorudinia, and J. Ghanavi. 2016. Effect of heart valve decellularization on xenograft rejection. *Experimental and Clinical Transplantation: Official Journal of the Middle East Society for Organ Transplantation*. doi:10.6002/ect.2015.0321.
- Ahmed, T. A. E., E. V. Dare, and M. Hincke. 2008. Fibrin: A versatile scaffold for tissue engineering applications. *Tissue Engineering. Part B, Reviews* 14 (2): 199–215. doi:10.1089/ten.teb.2007.0435.
- Akhyari, P., H. Kamiya, P. Gwanmesia, H. Aubin, R. Tschierschke, S. Hoffmann, M. Karck, and A. Lichtenberg. 2010. In vivo functional performance and structural maturation of decellularised allogenic aortic valves in the subcoronary position. *European Journal of Cardio-Thoracic Surgery* 38 (5): 539–546. doi:10.1016/j.ejcts.2010.03.024.
- Alavi, S. H., M. S. Baliarda, N. Bonessio, L. Valdevit, and A. Kheradvar. 2017. A tri-leaflet nitinol mesh scaffold for engineering heart valves. *Annals of Biomedical Engineering* 45 (2): 413–426. doi:10.1007/s10439-016-1778-0.
- Albanna, M. Z., T. H. Bou-Akl, H. L. Walters, and H. W. T. Matthew. 2012. Improving the mechanical properties of chitosan-based heart valve scaffolds using chitosan fibers. *Journal of the Mechanical Behavior of Biomedical Materials* 5 (1): 171–180. doi:10.1016/j.jmbbm.2011.08.021.
- Alfonso, A. R., S. Rath, P. Rafiee, M. Hernandez-Espino, M. Din, F. George, and S. Ramaswamy. 2013. Glycosaminoglycan entrapment by fibrin in engineered heart valve tissues. *Acta Biomaterialia* 9 (9): 8149–8157. doi:10.1016/j.actbio.2013.06.009.
- Amoroso, N. J., A. D'Amore, Y. Hong, C. P. Rivera, M. S. Sacks, and W. R. Wagner. 2012. Microstructural manipulation of electrospun scaffolds for specific bending stiffness for heart valve tissue engineering. *Acta Biomaterialia* 8 (12): 4268–4277. doi:10.1016/j.actbio.2012.08.002.
- Armstrong, E. J., and J. Bischoff. 2004. Heart valve development: Endothelial cell signaling and differentiation. *Circulation Research* 95 (5): 459–470. doi:10.1161/01.RES.0000141146.95728.da.
- Arnold, R., J. Ley-Zaporozhan, S. Ley, T. Loukanov, C. Sebening, J. B. Kleber, B. Goebel, S. Hagl, M. Karck, and M. Gorenflo. 2008. Outcome after mechanical aortic valve replacement in children and young adults. *The Annals of Thoracic Surgery* 85 (2): 604–610. doi:10.1016/j.athoracsur.2007.10.035.
- Azadani, A. N., S. Chitsaz, P. B. Matthews, N. Jaussaud, J. Leung, T. Tsinman, L. Ge, and E. E. Tseng. 2012a. Comparison of mechanical properties of human ascending aorta and aortic sinuses. *The Annals of Thoracic Surgery* 93 (1): 87–94. doi:10.1016/j.athoracsur.2011.08.002.
- Azadani, A. N., S. Chitsaz, P. B. Matthews, N. Jaussaud, J. Leung, A. Wisneski, L. Ge, and E. E. Tseng. 2012b. Biomechanical comparison of human pulmonary and aortic roots. *European Journal of Cardio-Thoracic Surgery* 41 (5): 1111–1116. doi:10.1093/ejcts/ezr163.
- Bäck, M., T. C. Gasser, J. B. Michel, and G. Caligiuri. 2013. Biomechanical Factors in the biology of aortic wall and aortic valve diseases. *Cardiovascular Research* 99 (2): 232–241. doi:10.1093/cvr/cvt040.

- Balachandran, K., P. Sucusky, and A. P. Yoganathan. 2011. Hemodynamics and mechanobiology of aortic valve inflammation and calcification. *International Journal of Inflammation* 2011: 263870. doi:10.4061/2011/263870.
- Balguid, A., M. P. Rubbens, A. Mol, R. A. Bank, A. J. Bogers, J. P. van Kats, B. A. de Mol, F. P. Baaijens, and C. V. Bouten. 2007. The role of collagen cross-links in biomechanical behavior of human aortic heart valve leaflets--relevance for tissue engineering. *Tissue Engineering* 13 (7): 1501–1511. doi:10.1089/ten.2006.0279.
- Benjamin, E. J., M. J. Blaha, S. E. Chiuve, M. Cushman, S. R. Das, R. Deo, S. D. de Ferranti et al. 2017. Heart disease and stroke statistics—2017 Update: A report from the American heart association. *Circulation*. doi:10.1161/CIR.0000000000000485.
- Bibevski, S., M. Ruzmetov, R. S. Fortuna, M. W. Turrentine, J. W. Brown, and R. G. Ohye. 2017. Performance of synergraft decellularized pulmonary allografts compared with standard cryopreserved allografts: Results from multiinstitutional data. *The Annals of Thoracic Surgery* 103 (3): 869–874. doi:10.1016/j.athoracsur.2016.07.068.
- Billiar, K. L., and M. S. Sacks. 1999. Biaxial mechanical properties of the natural and glutaraldehyde treated aortic valve cusp—Part I: Experimental results. *Journal of Biomechanical Engineering* 122 (1): 23–30. doi:10.1115/1.429624.
- Bloch, O., P. Golde, P. M. Dohmen, S. Posner, W. Konertz, and W. Erdbrügger. 2011. Immune response in patients receiving a bioprosthetic heart valve: Lack of response with decellularized valves. *Tissue Engineering. Part A* 17 (19–20): 2399–2405. doi:10.1089/ten. TEA.2011.0046.
- Breuer, C. K., T. Shin'oka, R. E. Tanel, G. Zund, D. J. Mooney, P. X. Ma, T. Miura et al. 1996. Tissue engineering lamb heart valve leaflets. *Biotechnology and Bioengineering* 50 (5): 562–567. doi:10.1002/(SICI)1097-0290(19960605)50:5 < 562::AID-BIT11 > 3.0.CO;2-L.
- Brougham, C. M., T. J. Levingstone, S. Jockenhoovel, T. C. Flanagan, and F. J. O'Brien. 2015. Incorporation of fibrin into a collagen-glycosaminoglycan matrix results in a scaffold with improved mechanical properties and enhanced capacity to resist cell-mediated contraction. *Acta Biomaterialia* 26: 205–14. doi:10.1016/j.actbio.2015.08.022.
- Brown, J. W., M. Ruzmetov, O. Eltayeb, M. D. Rodefeld, and M. W. Turrentine. 2011. Performance of synergraft decellularized pulmonary homograft in patients undergoing a ross procedure. *The Annals of Thoracic Surgery* 91 (2): 416–422–423. doi:10.1016/j.athoracsur.2010.10.069.
- Buchanan, R. M., and M. S. Sacks. 2014. Interlayer micromechanics of the aortic heart valve leaflet. *Biomechanics and Modeling in Mechanobiology* 13 (4): 813–826. doi:10.1007/s10237-013-0536-6.
- Butcher, J. T., G. J. Mahler, and L. A. Hockaday. 2011. Aortic valve disease and treatment: The need for naturally engineered solutions. *Advanced Drug Delivery Reviews* 63 (4–5): 242–268. doi:10.1016/j.addr.2011.01.008.
- Butcher, J. T., and R. R. Markwald. 2007. Valvulogenesis: The moving target. *Philosophical Transactions of the Royal Society B: Biological Sciences* 362 (1484): 1489–1503. doi:10.1098/rstb.2007.2130.
- Butcher, J. T., and R. M. Nerem. 2006. Valvular endothelial cells regulate the phenotype of interstitial cells in co-culture: Effects of steady shear stress. *Tissue Engineering* 12 (4): 905–915. doi:10.1089/ten.2006.12.905.
- Carapetis, J. R., A. C. Steer, E. K. Mulholland, and M. Weber. 2005. The global burden of group a streptococcal diseases. *The Lancet: Infectious Diseases* 5 (11): 685–694. doi:10.1016/S1473-3099(05)70267-X.
- Charitos, E. I., and H. H. Sievers. 2013. Anatomy of the aortic root: Implications for valve-sparing surgery. *Annals of Cardiothoracic Surgery* 2 (1): 53–56. doi:10.3978/j.issn.2225-319X.2012.11.18.

- Chaux, A., R. J. Gray, J. C. Stupka, M. R. Emken, L. N. Scotten, and R. Siegel. 2016. Anticoagulant independent mechanical heart valves: Viable now or still a distant holy grail. *Annals of Translational Medicine* 4 (24): 525. doi:10.21037/atm.2016.12.58.
- Chen, J. H., C. Y. Y. Yip, E. D. Sone, and C. A. Simmons. 2009. Identification and characterization of aortic valve mesenchymal progenitor cells with robust osteogenic calcification potential. *American Journal of Pathology* 174: 1109–1119.
- Cheung, D. Y., B. Duan, and J. T. Butcher. 2015. Current progress in tissue engineering of heart valves: Multiscale problems, multiscale solutions. *Expert Opinion on Biological Therapy* 15 (8): 1155–1172. doi:10.1517/14712598.2015.1051527.
- Chiang, Y. P., J. Chikwe, A. J. Moskowitz, S. Itagaki, D. H. Adams, and N. N. Egorova. 2014. Survival and long-term outcomes following bioprosthetic vs mechanical aortic valve replacement in patients aged 50 to 69 years. *JAMA* 312 (13): 1323–1329. doi:10.1001/jama.2014.12679.
- Chu, P. K., J. Y. Chen, L. P. Wang, and N. Huang. 2002. Plasma-surface modification of biomaterials. *Materials Science and Engineering: R: Reports* 36 (5–6): 143–206. doi:10.1016/S0927-796X(02)00004-9.
- Cicha, I., A. Rüffer, R. Cesnjevar, M. Glöckler, A. Agaimy, W. G. Daniel, C. D. Garlichs, and S. Dittrich. 2011. Early obstruction of decellularized xenogenic valves in pediatric patients: Involvement of inflammatory and fibroproliferative processes. *Cardiovascular Pathology: The Official Journal of the Society for Cardiovascular Pathology* 20 (4): 222–231. doi:10.1016/j.carpath.2010.04.006.
- Claiborne, T. E., M. J. Slepian, S. Hossainy, and D. Bluestein. 2012. Polymeric trileaflet prosthetic heart valves: Evolution and path to clinical reality. *Expert Review of Medical Devices* 9 (6): 577–594. doi:10.1586/erd.12.51.
- Crapo, P. M., T. W. Gilbert, and S. F. Badylak. 2011. An overview of tissue and whole organ decellularization processes. *Biomaterials* 32 (12): 3233–3243. doi:10.1016/j.biomaterials.2011.01.057.
- Dagum, P., G. R. Green, F. J. Nistal, G. T. Daughters, T. A. Timek, L. E. Foppiano, A. F. Bolger, N. B. Ingels, and D. C. Miller. 1999. Deformational dynamics of the aortic root modes and physiologic determinants. *Circulation* 100 (suppl 2): II-54–II-62. doi:10.1161/01.CIR.100.suppl_2.II-54.
- Dijkman, P. E., A. Driessen-Mol, L. Frese, S. P. Hoerstrup, and F. P. T. Baaijens. 2012. Decellularized homologous tissue-engineered heart valves as off-the-shelf alternatives to xeno- and homografts. *Biomaterials* 33 (18): 4545–4554. doi:10.1016/j.biomaterials.2012.03.015.
- Dohmen, P. M., F. da Costa, S. Holinski, S. V. Lopes, S. Yoshi, L. H. Reichert, R. Villani, S. Posner, and W. Konertz. 2006a. Is there a possibility for a glutaraldehyde-free porcine heart valve to grow? *European Surgical Research* 38 (1): 54–61. doi:10.1159/000091597.
- Dohmen, P. M., F. da Costa, S. Yoshi, S. V. Lopes, F. P. da Souza, R. Vilani, A. F. Wouk, M. da Costa, and W. Konertz. 2006b. Histological evaluation of tissue-engineered heart valves implanted in the juvenile sheep model: Is there a need for in-vitro seeding? *The Journal of Heart Valve Disease* 15 (6): 823–829.
- Driessen-Mol, A., M. Y. Emmert, P. E. Dijkman, L. Frese, B. Sanders, B. Weber, N. Cesarovic et al. 2014. Transcatheter implantation of homologous ‘off-the-shelf’ tissue-engineered heart valves with self-repair capacity: Long-term functionality and rapid in vivo remodeling in sheep. *Journal of the American College of Cardiology* 63 (13): 1320–1329. doi:10.1016/j.jacc.2013.09.082.
- Duan, B., L. A. Hockaday, K. H. Kang, and J. T. Butcher. 2013. 3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *Journal of Biomedical Materials Research. Part A* 101 (5): 1255–1264. doi:10.1002/jbm.a.34420.

- Duan, B., L. A. Hockaday, E. Kapetanovic, K. H. Kang, and J. T. Butcher. 2013. Stiffness and adhesivity control aortic valve interstitial cell behavior within hyaluronic acid-based hydrogels. *Acta Biomaterialia* 9 (8): 7640–7650. doi:10.1016/j.actbio.2013.04.050.
- Duan, B., E. Kapetanovic, L. A. Hockaday, and J. T. Butcher. 2014. Three-dimensional printed rrileaflet valve conduits using iological hydrogels and human valve interstitial cells. *Acta Biomaterialia* 10 (5): 1836–1846. doi:10.1016/j.actbio.2013.12.005.
- Dubé, J., J. M. Bourget, R. Gauvin, H. Lafrance, C. J. Roberge, F. A. Auger, and L. Germain. 2014. Progress in developing a living human tissue-engineered tri-leaflet heart valve assembled from tissue produced by the self-assembly approach. *Acta Biomaterialia* 10 (8): 3563–3570. doi:10.1016/j.actbio.2014.04.033.
- Eckert, C. E., R. Fan, B. Mikulis, M. Barron, C. A. Carruthers, V. M. Friebe, N. R. Vyavahare, and M. S. Sacks. 2013. On the biomechanical role of glycosaminoglycans in the aortic heart valve leaflet. *Acta Biomaterialia* 9 (1): 4653–4660. doi:10.1016/j.actbio.2012.09.031.
- Eckert, C. E., B. T. Mikulis, D. Gottlieb, D. Gerneke, I. LeGrice, R. F. Padera, J. E. Mayer, F. J. Schoen, and M. S. Sacks. 2011. Three-dimensional quantitative micromorphology of pre- and post-implanted engineered heart valve tissues. *Annals of Biomedical Engineering* 39 (1): 205–222. doi:10.1007/s10439-010-0162-8.
- Eslami, M., N. E. Vrana, P. Zorlutuna, S. Sant, S. Jung, N. Masoumi, R. A. Khavari-Nejad, G. Javadi, and A. Khademhosseini. 2014. Fiber-reinforced hydrogel scaffolds for heart valve tissue engineering. *Journal of Biomaterials Applications* 29 (3): 399–410. doi:10.1177/0885328214530589.
- Etnel, J. R., L. C. Elmont, E. Ertekin, M. M. Mokhles, H. J. Heuvelman, J. W. Roos-Hesselink, P. L. de Jong, W. A. Helbing, A. J. Bogers, and J. J. Takkenberg. 2016. Outcome after aortic valve replacement in children: A systematic review and meta-analysis. *The Journal of Thoracic and Cardiovascular Surgery* 151 (1): 143–152. doi:10.1016/j.jtcvs.2015.09.083.
- Fan, R., A. S. Bayoumi, P. Chen, C. M. Hobson, W. R. Wagner, J. E. Mayer, and M. S. Sacks. 2013. Optimal elastomeric scaffold leaflet shape for pulmonary heart valve leaflet replacement. *Journal of Biomechanics* 46 (4): 662–669. doi:10.1016/j.jbiomech.2012.11.046.
- Farrar, E. J., V. Pramit, J. M. Richards, C. Z. Mosher, and J. T. Butcher. 2016. Valve interstitial cell tensional homeostasis directs calcification and extracellular matrix remodeling processes via RhoA signaling. *Biomaterials* 105: 25–37. doi:10.1016/j.biomaterials.2016.07.034.
- Feng, Z., Y. Wagatsuma, M. Kikuchi, T. Kosawada, T. Nakamura, D. Sato, N. Shirasawa, T. Kitajima, and M. Umez. 2014. The mechanisms of fibroblast-mediated compaction of collagen gels and the mechanical niche around individual fibroblasts. *Biomaterials* 35 (28): 8078–8091. doi:10.1016/j.biomaterials.2014.05.072.
- Flameng, W., G. De Visscher, L. Mesure, H. Hermans, R. Jashari, and B. Meuris. 2014. Coating with fibronectin and stromal cell-derived factor- 1 α of decellularized homografts used for right ventricular outflow tract reconstruction eliminates immune response-related degeneration. *Journal of Thoracic and Cardiovascular Surgery* 147 (4): 1398–1404.e2. doi:10.1016/j.jtcvs.2013.06.022.
- Flanagan, T. C., C. Cornelissen, S. Koch, B. Tschoeke, J. S. Sachweh, T. Schmitz-Rode, and S. Jockenhoevel. 2007. The in vitro development of autologous dibrin-based tissue-engineered heart valves through optimised dynamic conditioning. *Biomaterials* 28 (23): 3388–3397. doi:10.1016/j.biomaterials.2007.04.012.
- Flanagan, T. C., J. S. Sachweh, J. Frese, H. Schnöring, N. Gronloh, S. Koch, R. H. Tolba, T. Schmitz-Rode, and S. Jockenhoevel. 2009. *In Vivo* remodeling and structural characterization of fibrin-based tissue-engineered heart valves in the adult sheep model. *Tissue Engineering Part A* 15 (10): 2965–2976. doi:10.1089/ten.tea.2009.0018.

- Frendl, C. M., S. M. Tucker, N. A. Khan, M. B. Esch, S. Kanduru, T. M. Cao, A. J. García, M. R. King, and J. T. Butcher. 2014. Endothelial retention and phenotype on carbonized cardiovascular implant surfaces. *Biomaterials* 35 (27): 7714–7723. doi:10.1016/j.biomaterials.2014.05.075.
- Friedrich, L. H., P. Jungebluth, S. Sjoqvist et al. 2014. Preservation of aortic root architecture and properties using a detergent-enzymatic perfusion protocol. *Biomaterials* 35 (6): 1907–1913. doi:10.1016/j.biomaterials.2013.11.053.
- Fu, J. H., M. Zhao, Y. R. Lin, X. D. Tian, Y. D. Wang, Z. X. Wang, and L. X. Wang. 2017. Degradable chitosan-collagen composites seeded with cells as tissue engineered heart valves. *Heart, Lung & Circulation* 26 (1): 94–100. doi:10.1016/j.hlc.2016.05.116.
- Funayama, M., Y. Takewa, T. Oie, Y. Matsui, E. Tatsumi, and Y. Nakayama. 2015. In situ observation and enhancement of leaflet tissue formation in bioprosthetic ‘biovalve.’ *Journal of Artificial Organs* 18 (1): 40–47. doi:10.1007/s10047-014-0793-x.
- Gaharwar, A. K., M. Nikkhah, S. Sant, and A. Khademhosseini. 2014. Anisotropic poly (glycerol sebacate)-poly (ϵ -caprolactone) electrospun fibers promote endothelial cell guidance. *Biofabrication* 7 (1): 15001. doi:10.1088/1758-5090/7/1/015001.
- Gallegos, R. P., A. L. Rivard, P. T. Suwan, S. Black, S. Bertog, U. Steinseifer, A. Armien, M. Lahti, and R. W. Bianco. 2006. In-vivo experience with the triflo trileaflet mechanical heart valve. *The Journal of Heart Valve Disease* 15 (6): 791–799.
- Gallyamov, M. O., I. S. Chaschin, M. A. Khokhlova, T. E. Grigorev, N. P. Bakuleva, I. G. Lyutova, J. E. Kondratenko, G. A. Badun, M. G. Chernysheva, and A. R. Khokhlov. 2014. Collagen tissue treated with chitosan solutions in carbonic acid for improved biological prosthetic heart valves. *Materials Science and Engineering: C* 37: 127–140. doi:10.1016/j.msec.2014.01.017.
- Gerstle, T. L., A. M. S. Ibrahim, P. S. Kim, B. T. Lee, and S. J. Lin. 2014. A plastic surgery application in evolution: Three-dimensional printing. *Plastic and Reconstructive Surgery* 133 (2): 446–451. doi:10.1097/01.prs.0000436844.92623.d3.
- Ghanbari, H., H. Viatge, A. G. Kidane, G. Burriesci, M. Tavakoli, and A. M. Seifalian. 2009. Polymeric heart valves: New materials, emerging hopes. *Trends in Biotechnology* 27 (6): 359–367. doi:10.1016/j.tibtech.2009.03.002.
- Glaser, N., V. Jackson, M. J. Holzmann, A. Franco-Cereceda, and U. Sartipy. 2015. Aortic valve replacement with mechanical vs. biological prostheses in patients aged 50–69 years. *European Heart Journal*. doi:10.1093/eurheartj/ehv580.
- Go, A. S., D. Mozaffarian, V. L. Roger, E. J. Benjamin, J. D. Berry, W. B. Borden, D. M. Bravata et al. 2013. Heart disease and stroke statistics—2013 update a report from the American heart association. *Circulation* 127 (1): e6–e245. doi:10.1161/CIR.0b013e31828124ad.
- Gottlieb, D., T. Kunal, S. Emani, E. Aikawa, D. W. Brown, A. J. Powell, A. Nedder et al. 2010. In vivo monitoring of function of autologous engineered pulmonary valve. *The Journal of Thoracic and Cardiovascular Surgery* 139 (3): 723–731. doi:10.1016/j.jtcvs.2009.11.006.
- Hammermeister, K., G. K. Sethi, W. G. Henderson, F. L. Grover, C. Oprian, and S. H. Rahimtoola. 2000. Outcomes 15 years after valve replacement with a mechanical versus a bioprosthetic valve: Final report of the veterans affairs randomized trial. *Journal of the American College of Cardiology* 36 (4): 1152–1158. doi:10.1016/S0735-1097(00)00834-2.
- Hasan, A., J. Saliba, H. P. Modarres, A. Bakhaty, A. Nasajpour, M. R. K. Mofrad, and A. Sanati-Nezhad. 2016. Micro and nanotechnologies in heart valve tissue engineering. *Biomaterials* 103: 278–292. doi:10.1016/j.biomaterials.2016.07.001.
- Hinderer, S., J. Seifert, M. Votteler et al. 2014. Engineering of a bio-functionalized hybrid off-the-shelf heart valve. *Biomaterials* 35 (7): 2130–2139. doi:10.1016/j.biomaterials.2013.10.080.

- Ho, S. Y. 2009. Structure and anatomy of the aortic root. *European Heart Journal-Cardiovascular Imaging* 10 (1): i3–10. doi:10.1093/ejehocardi/jen243.
- Hobson, C. M., N. J. Amoroso, R. Amini, E. Ungchusri, Y. Hong, A. D'Amore, M. S. Sacks, and W. R. Wagner. 2015. Fabrication of elastomeric scaffolds with curvilinear fibrous structures for heart valve leaflet engineering. *Journal of Biomedical Materials Research. Part A* 103 (9): 3101–3106. doi:10.1002/jbm.a.35450.
- Hockaday, L. A., B. Duan, K. H. Kang, and J. T. Butcher. 2014. 3D-printed hydrogel technologies for tissue-engineered heart valves. *3D Printing and Additive Manufacturing* 1 (3): 122–136. doi:10.1089/3dp.2014.0018.
- Hockaday, L. A., K. H. Kang, N. W. Colangelo, P. Y. C. Cheung, B. Duan, E. Malone, J. Wu et al. 2012. Rapid 3D printing of anatomically accurate and mechanically heterogeneous aortic valve hydrogel scaffolds. *Biofabrication* 4 (3): 35005. doi:10.1088/1758-5082/4/3/035005.
- Hoerstrup, S. P., R. Sodian, S. Daebritz, J. Wang, E. A. Bacha, D. P. Martin, A. M. Moran et al. 2000. Functional living trileaflet heart valves grown in vitro. *Circulation* 102 (suppl 3): Iii-44–Iii-49. doi:10.1161/01.CIR.102.suppl_3.III-44.
- Iliopoulos, D. C., E. P. Kritharis, S. Boussias, A. Demis, C. D. Iliopoulos, and D. P. Sokolis. 2013. Biomechanical properties and histological structure of sinus of valsalva aneurysms in relation to age and region. *Journal of Biomechanics* 46 (5): 931–940. doi:10.1016/j.jbiomech.2012.12.004.
- Jahnavi, S., T. V. Kumary, G. S. Bhuvaneshwar, T. S. Natarajan, and R. S. Verma. 2015. Engineering of a polymer layered bio-hybrid heart valve scaffold. *Materials Science & Engineering. C, Materials for Biological Applications* 51: 263–273. doi:10.1016/j.msec.2015.03.009.
- Jahnavi, S. et al. 2017. Biological and mechanical evaluation of a Bio-Hybrid scaffold for autologous valve tissue engineering. *Materials Science and Engineering: C* 73: 59–71.
- Jana, S., B. J. Tefft, D. B. Spoon, and R. D. Simari. 2014. Scaffolds for tissue engineering of cardiac valves. *Acta Biomaterialia* 10 (7): 2877–2893. doi:10.1016/j.actbio.2014.03.014.
- Jordan, J. E., J. K. Williams, S.-J. Lee, D. Raghavan, A. Atala, and J. J. Yoo. 2012. Bioengineered self-seeding heart valves. *Journal of Thoracic and Cardiovascular Surgery* 143 (1): 201–208. doi:10.1016/j.jtcvs.2011.10.005.
- Kallio, M., J. Pihkala, H. Sairanen, and I. Mattila. 2015. Long-term results of the ross procedure in a population-based follow-up. *European Journal of Cardio-Thoracic Surgery: Official Journal of the European Association for Cardio-Thoracic Surgery* 47 (5): e164–e170. doi:10.1093/ejcts/ezv004.
- Karaskov, A., R. Sharifulin, S. Zheleznev, I. Demin, E. Lenko, and A. Bogachev-Prokophiev. 2016. Results of the ross procedure in adults: A single-centre experience of 741 operations. *European Journal of Cardio-Thoracic Surgery* 49 (5): e97–e104. doi:10.1093/ejcts/ezw047.
- Kasel, A. M., S. Cassese, S. Bleiziffer, M. Amaki, R. T. Hahn, A. Kastrati, and P. P. Sengupta. 2013. Standardized imaging for aortic annular sizing: Implications for transcatheter valve selection. *JACC. Cardiovascular Imaging* 6 (2): 249–262. doi:10.1016/j.jcmg.2012.12.005.
- Kasimir, M. T., G. Weigel, J. Sharma, E. Rieder, G. Seebacher, E. Wolner, and P. Simon. 2005. The decellularized porcine heart valve matrix in tissue engineering: Platelet adhesion and activation. *Thrombosis and Haemostasis* 94 (3): 562–567. doi:10.1160/TH05-01-0025.
- Kishimoto, S., Y. Takewa, Y. Nakayama, K. Date, H. Sumikura, T. Moriwaki, M. Nishimura, and E. Tatsumi. 2015. Sutureless aortic valve replacement using a novel autologous tissue heart valve with stent (stent biovalve): Proof of concept. *Journal of Artificial Organs: The Official Journal of the Japanese Society for Artificial Organs*. doi:10.1007/s10047-015-0817-1.

- Kluin, J., H. Talacua, A. I. P. M. Smits, M. Y. Emmert, M. C. P. Brugmans, E. S. Fioretta, P. E. Dijkman et al. 2017. In situ heart valve tissue engineering using a bioresorbable elastomeric implant—from material design to 12 months follow-up in sheep. *Biomaterials* 125: 101–117. doi:10.1016/j.biomaterials.2017.02.007.
- Kneib, C., C. Q. C. von Glehn, F. D. A. Costa, M. T. B. A. Costa, and M. F. Susin. 2012. Evaluation of humoral immune response to donor HLA after implantation of cellularized versus decellularized human heart valve allografts. *Tissue Antigens* 80 (2): 165–174. doi:10.1111/j.1399-0039.2012.01885.x.
- Konertz, W., P. M. Dohmen, J. Liu, S. Beholz, S. Dushe, S. Posner, A. Lembcke, and W. Erdbrügger. 2005. Hemodynamic characteristics of the matrix P decellularized xenograft for pulmonary valve replacement during the ross operation. *The Journal of Heart Valve Disease* 14 (1): 78–81.
- Labaf, A., P. J. Svensson, H. Renlund, A. Jeppsson, and A. Sjölander. 2016. Incidence and risk factors for thromboembolism and major bleeding in patients with mechanical valve prosthesis: A nationwide population-based study. *American Heart Journal* 181: 1–9. doi:10.1016/j.ahj.2016.06.026.
- Latif, N., P. Sarathchandra, P. M. Taylor, J. Antoniwi, and M. H. Yacoub. 2005. Localization and pattern of expression of extracellular matrix components in human heart valves. *The Journal of Heart Valve Disease* 14 (2): 218–227.
- Lee, J. M., D. W. Courtman, and D. R. Boughner. 1984. The glutaraldehyde-stabilized porcine aortic valve xenograft. I. tensile viscoelastic properties of the fresh leaflet material. *Journal of Biomedical Materials Research* 18 (1): 61–77. doi:10.1002/jbm.820180108.
- Liao, J., E. M. Joyce, and M. S. Sacks. 2008. Effects of decellularization on the mechanical and structural properties of the porcine aortic valve leaflet. *Biomaterials* 29 (8): 1065–1074. doi:10.1016/j.biomaterials.2007.11.007.
- Lim, H.-G., G. B. Kim, S. Jeong, and Y. J. Kim. 2014. Development of a next-generation tissue valve using a glutaraldehyde-fixed porcine aortic valve treated with decellularization, α -galactosidase, space filler, organic solvent and detoxification. *European Journal of Cardio-Thoracic Surgery* 48: 101–113. doi:10.1093/ejcts/ezu385.
- Liu, A. C., V. R. Joag, and A. I. Gotlieb. 2007. The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. *The American Journal of Pathology* 171 (5): 1407–1418. doi:10.2353/ajpath.2007.070251.
- Loosdregt, I. A. E. W. van, G. Argento, A. Driessen-Mol, C. W. J. Oomens, and F. P. T. Baaijens. 2014. Cell-mediated retraction versus hemodynamic loading—a delicate balance in tissue-engineered heart valves. *Journal of Biomechanics* 47 (9): 2064–2069. doi:10.1016/j.jbiomech.2013.10.049.
- López-Mínguez, J. R., V. Climent, S. Yen-Ho, R. González-Fernández, J. M. Nogales-Asensio, and D. Sánchez-Quintana. 2006. Structural features of the sinus of valsalva and the proximal portion of the coronary arteries: Their relevance to retrograde aortocoronary dissection. *Revista Española de Cardiología (English Edition)* 59 (7): 696–702. doi:10.1016/S1885-5857(07)60028-0.
- Lueders, C., B. Jastram, R. Hetzer, and H. Schwandt. 2014. Rapid manufacturing techniques for the tissue engineering of human heart valves. *European Journal of Cardio-Thoracic Surgery* 46 (4): 593–601. doi:10.1093/ejcts/ezt510.
- MacGrogan, D., G. Luxán, A. Driessen-Mol, C. Bouten, F. Baaijens, and J. L. de la Pompa. 2014. How to make a heart valve: From embryonic development to bioengineering of living valve substitutes. *Cold Spring Harbor Perspectives in Medicine* 4 (11): a013912. doi:10.1101/cshperspect.a013912.

- Mahler, G. J., E. J. Farrar, and J. T. Butcher. 2013. Inflammatory cytokines promote mesenchymal transformation in embryonic and adult valve endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 33 (1): 121–130. doi:10.1161/ATVBAHA.112.300504.
- Masjedi, S., A. Amarnath, K. M. Baily, and Z. Ferdous. 2016. Comparison of calcification potential of valvular interstitial cells isolated from individual aortic valve cusps. *Cardiovascular Pathology: The Official Journal of the Society for Cardiovascular Pathology* 25 (3): 185–194. doi:10.1016/j.carpath.2015.12.002.
- Masoumi, N., N. Annabi, A. Assmann, B. L. Larson, J. Hjortnaes, N. Alemdar, M. Kharaziha, K. B. Manning, J. E. Mayer, and A. Khademhosseini. 2014a. Tri-layered elastomeric scaffolds for engineering heart valve leaflets. *Biomaterials* 35 (27): 7774–7785. doi:10.1016/j.biomaterials.2014.04.039.
- Masoumi, N., K. L. Johnson, M. C. Howell, and G. C. Engelmayr Jr. 2013. Valvular interstitial cell seeded poly(glycerol sebacate) scaffolds: Toward a biomimetic in vitro model for heart valve tissue engineering. *Acta Biomaterialia* 9 (4): 5974–5988. doi:10.1016/j.actbio.2013.01.001.
- Masoumi, N., B. L. Larson, N. Annabi, M. Kharaziha, B. Zamanian, K. S. Shapero, A. T. Cubberley et al. 2014b. Electrospun PGS: PCL microfibers align human valvular interstitial cells and provide tunable scaffold anisotropy. *Advanced Healthcare Materials* 3 (6): 929–939. doi:10.1002/adhm.201300505.
- Masuda, M., H. Kado, Y. Ando, A. Shiose, T. Nakano, K. Fukae, Y. Tanoue, and R. Tominaga. 2008. Intermediate-term results after the aortic valve replacement using bileaflet mechanical prosthetic valve in children. *European Journal of Cardio-Thoracic Surgery: Official Journal of the European Association for Cardio-Thoracic Surgery* 34 (1): 42–47. doi:10.1016/j.ejcts.2008.04.005.
- Miragoli, M., M. H. Yacoub, I. El-Hamamsy, J. L. Sanchez-Alonso, A. Moshkov, N. Mongkoldhumrongkul, M. Padala et al. 2014. Side-specific mechanical properties of valve endothelial cells. *American Journal of Physiology: Heart and Circulatory Physiology* 307 (1): H15–H24. doi:10.1152/ajpheart.00228.2013.
- Misawa, Y. 2014. Valve-related complications after mechanical heart valve implantation. *Surgery Today*. doi:10.1007/s00595-014-1104-0.
- Muraru, D., F. Veronesi, A. Maddalozzo, D. Dequal, L. Frajhof, A. Rabischowsky, S. Iliceto, and L. P. Badano. 2016. 3D printing of normal and pathologic tricuspid valves from trans-thoracic 3D echocardiography data sets. *European Heart Journal of Cardiovascular Imaging*, jew215. doi:10.1093/ehjci/jew215.
- Murphy, S. V., and A. Atala. 2014. 3D bioprinting of tissues and organs. *Nature Biotechnology* 32 (8): 773–785. doi:10.1038/nbt.2958.
- Namdari, M., and A. Eatemadi. 2016. Nanofibrous bioengineered heart valve-application in paediatric medicine. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie* 84: 1179–1188. doi:10.1016/j.biopha.2016.10.058.
- Neidert, M. R., and R. T. Tranquillo. 2006. Tissue-engineered valves with commissural alignment. *Tissue Engineering* 12 (4): 891–903. doi:10.1089/ten.2006.12.891.
- Neumann, A., S. Sarikouch, T. Breyman, S. Cebotari, D. Boethig, A. Horke, P. Beerbaum et al. 2014. Early systemic cellular immune response in children and young adults receiving decellularized fresh allografts for pulmonary valve replacement. *Tissue Engineering. Part A* 20 (5–6): 1003–1011. doi:10.1089/ten.tea.2013.0316.
- Nishimura, R. A. 2002. Aortic valve disease. *Circulation* 106 (7): 770–772. doi:10.1161/01.CIR.0000027621.26167.5E.
- Nomoto, R., L. A. Sleeper, M. J. Borisuk, L. Bergerson, F. A. Pigula, S. Emani, F. Fynn-Thompson, J. E. Mayer, P. J. Del Nido, and C. W. Baird. 2016. Outcome and performance of bioprosthetic pulmonary valve replacement in patients with congenital heart disease. *The Journal of Thoracic and Cardiovascular Surgery* 152 (5): 1333–1342.e3. doi:10.1016/j.jtcvs.2016.06.064.

- Okamoto, Y., K. Yamamoto, and S. Yoshii. 2016. Early and late outcomes of aortic valve replacement using bioprosthetic versus mechanical valve in elderly patients: A propensity analysis. *Journal of Cardiac Surgery* 31 (4): 195–202. doi:10.1111/jocs.12719.
- Paniagua Gutierrez, J. R., H. Berry, S. Korossis, S. Mirsadraee, S. V. Lopes, F. da Costa, J. Kearney, K. Watterson, J. Fisher, and E. Ingham. 2015. Regenerative potential of low-concentration SDS-decellularized porcine aortic valved conduits in vivo. *Tissue Engineering Part A* 21 (1–2): 332–342. doi:10.1089/ten.tea.2014.0003.
- Peltola, S. M., F. P. W. Melchels, D. W. Grijpma, and M. Kellomäki. 2008. A review of rapid prototyping techniques for tissue engineering purposes. *Annals of Medicine* 40 (4): 268–280. doi:10.1080/078553890701881788.
- Pérez, R. A., J. E. Won, J. C. Knowles, and H. W. Kim. 2013. Naturally and synthetic smart composite biomaterials for tissue regeneration. *Advanced Drug Delivery Reviews* 65 (4): 471–496. doi:10.1016/j.addr.2012.03.009.
- Perri, G., A. Polito, C. Esposito, S. B. Albanese, P. Francalanci, G. Pongiglione, and A. Carotti. 2012. Early and late failure of tissue-engineered pulmonary valve conduits used for right ventricular outflow tract reconstruction in patients with congenital heart disease. *European Journal of Cardio-Thoracic Surgery: Official Journal of the European Association for Cardio-Thoracic Surgery* 41 (6): 1320–1325. doi:10.1093/ejcts/ezr221.
- Pho, M., W. Lee, D. R. Watt, C. Laschinger, C. A. Simmons, and C. A. McCulloch. 2008. Cofilin is a marker of myofibroblast differentiation in cells from porcine aortic cardiac valves. *American Journal of Physiology-Heart and Circulatory Physiology* 294 (4): H1767–H1778. doi:10.1152/ajpheart.01305.2007.
- Pibarot, P., and J. G. Dumesnil. 2009. Prosthetic heart valves selection of the optimal prosthesis and long-term management. *Circulation* 119 (7): 1034–1048. doi:10.1161/CIRCULATIONAHA.108.778886.
- Picard-Deland, M., J. Ruel, T. Galbraith, C. Tremblay, F. Kawecki, L. Germain, and F. A. Auger. 2016. Tissue-engineered tubular heart valves combining a novel precontraction phase with the self-assembly method. *Annals of Biomedical Engineering*. doi:10.1007/s10439-016-1708-1.
- Ramamurthi, A., and I. Vesely. 2005. Evaluation of the matrix-synthesis potential of cross-linked hyaluronan gels for tissue engineering of aortic heart valves. *Biomaterials* 26 (9): 999–1010. doi:10.1016/j.biomaterials.2004.04.016.
- Reimer, J., Z. Syedain, B. Haynie, M. Lahti, J. Berry, and R. Tranquillo. 2017. Implantation of a tissue-engineered tubular heart valve in growing lambs. *Annals of Biomedical Engineering* 45 (2): 439–451. doi:10.1007/s10439-016-1605-7.
- Reimer, J. M., Z. H. Syedain, B. H. T. Haynie, and R. T. Tranquillo. 2015. Pediatric tubular pulmonary heart valve from decellularized engineered tissue tubes. *Biomaterials* 62: 88–94. doi:10.1016/j.biomaterials.2015.05.009.
- Reineke, D., F. Gisler, L. Englberger, and T. Carrel. 2016. Mechanical versus biological aortic valve replacement strategies. *Expert Review of Cardiovascular Therapy* 14 (4): 423–430. doi:10.1586/14779072.2016.1133293.
- Roosens, A., P. Somers, F. De Somer, V. Carriel, G. Van Nooten, and R. Cornelissen. 2016. Impact of detergent-based decellularization methods on porcine tissues for heart valve engineering. *Annals of Biomedical Engineering* 44 (9): 2827–2839. doi:10.1007/s10439-016-1555-0.
- Ropcke, D. M. et al. 2017. Small intestinal submucosa tricuspid valve tube graft shows growth potential, remodeling and physiological valve function in a porcine model. *Interactive Cardiovascular and Thoracic Surgery* 24 (6): 918–924. doi:10.1093/icvts/ivx017.
- Sacks, M. S., and A. P. Yoganathan. 2007. Heart valve function: A biomechanical perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences* 362 (1484): 1369–1391. doi:10.1098/rstb.2007.2122.

- Sanders, B., S. Loerakker, E. S. Fioretta, D. J. P. Bax, A. Driessen-Mol, S. P. Hoerstrup, and F. P. T. Baaijens. 2016. Improved geometry of decellularized tissue engineered heart valves to prevent leaflet retraction. *Annals of Biomedical Engineering* 44: 1061–1071. doi:10.1007/s10439-015-1386-4.
- Sarikouch, S., A. Horke, I. Tudorache, P. Beerbaum, M. Westhoff-Bleck, D. Boethig, O. Repin et al. 2016. Decellularized fresh homografts for pulmonary valve replacement: A decade of clinical experience. *European Journal of Cardio-Thoracic Surgery: Official Journal of the European Association for Cardio-Thoracic Surgery*. doi:10.1093/ejcts/ezw050.
- Schmauss, D., C. Schmitz, A. K. Bigdeli, S. Weber, N. Gerber, A. Beiras-Fernandez, F. Schwarz, C. Becker, C. Kupatt, and R. Sodian. 2012. Three-dimensional printing of models for preoperative planning and simulation of transcatheter valve replacement. *The Annals of Thoracic Surgery* 93 (2): e31–e33. doi:10.1016/j.athoracsur.2011.09.031.
- Sewell-Loftin, M. K., Y. W. Chun, A. Khademhosseini, and W. D. Merryman. 2011. EMT-inducing biomaterials for heart valve engineering: Taking cues from developmental biology. *Journal of Cardiovascular Translational Research* 4 (5): 658–671. doi:10.1007/s12265-011-9300-4.
- Sharabiani, M. T. A., D. M. Dorobantu, A. S. Mahani, M. Turner, A. J. Peter Tometzki, G. D. Angelini, A. J. Parry, M. Caputo, and S. C. Stoica. 2016. Aortic valve replacement and the ross operation in children and young adults. *Journal of the American College of Cardiology* 67 (24): 2858–2870. doi:10.1016/j.jacc.2016.04.021.
- Silberman, S., A. Oren, M. Dotan, O. Merin, D. Fink, M. Deeb, and D. Bitran. 2008. Aortic valve replacement: Choice between mechanical valves and bioprostheses. *Journal of Cardiac Surgery* 23 (4): 299–306. doi:10.1111/j.1540-8191.2008.00580.x.
- Simon, P., M. T. Kasimir, G. Seebacher, G. Weigel, R. Ullrich, U. Salzer-Muhar, E. Rieder, and E. Wolner. 2003. Early failure of the tissue engineered porcine heart valve SYNERGRAFT® in pediatric patients. *European Journal of Cardio-Thoracic Surgery* 23 (6): 1002–1006.
- Singhal, P., A. Luk, and J. Butany. 2013. Bioprosthetic heart valves: Impact of implantation on biomaterials. *International Scholarly Research Notices* 2013: e728791. doi:10.5402/2013/728791.
- Sodian, R., S. P. Hoerstrup, J. S. Sperling, S. Daebritz, D. P. Martin, A. M. Moran, B. S. Kim, F. J. Schoen, J. P. Vacanti, and J. E. Mayer. 2000. Early in vivo experience with tissue-engineered trileaflet heart valves. *Circulation* 102 (suppl 3): Iii-22–Iii-29. doi:10.1161/01.CIR.102.suppl_3.III-22.
- Sodian, R., S. P. Hoerstrup, J. S. Sperling, S. H. Daebritz, D. P. Martin, F. J. Schoen, J. P. Vacanti, and J. E. Mayer. 2000. Tissue engineering of heart valves: In vitro experiences. *The Annals of Thoracic Surgery* 70 (1): 140–144.
- Sodian, R., S. P. Hoerstrup, J. S. Sperling, D. P. Martin, S. Daebritz, J. E. Mayer, and J. P. Vacanti. 2000. Evaluation of biodegradable, three-dimensional matrices for tissue engineering of heart valves. *ASAIO Journal (American Society for Artificial Internal Organs: 1992)* 46 (1): 107–110.
- Sodian, R., D. Schmauss, M. Markert, S. Weber, K. Nikolaou, S. Haeberle, F. Vogt et al. 2008. Three-dimensional printing creates models for surgical planning of aortic valve replacement after previous coronary bypass grafting. *The Annals of Thoracic Surgery* 85 (6): 2105–2108. doi:10.1016/j.athoracsur.2007.12.033.
- Sodian, R., D. Schmauss, C. Schmitz, A. Bigdeli, S. Haeberle, M. Schmoeckel, M. Markert et al. 2009. 3-dimensional printing of models to create custom-made devices for coil embolization of an anastomotic leak after aortic arch replacement. *The Annals of Thoracic Surgery* 88 (3): 974–978. doi:10.1016/j.athoracsur.2009.03.014.

- Sodian, R., J. S. Sperling, D. P. Martin, U. Stock, J. E. Mayer, and J. P. Vacanti. 1999. Tissue engineering of a trileaflet heart valve-early in vitro experiences with a combined polymer. *Tissue Engineering* 5 (5): 489–494.
- Sodian, R., S. Weber, M. Markert, M. Loeff, T. Lueth, F. C. Weis, S. Daebritz, E. Malec, C. Schmitz, and B. Reichart. 2008. Pediatric cardiac transplantation: Three-dimensional printing of anatomic models for surgical planning of heart transplantation in patients with univentricular heart. *The Journal of Thoracic and Cardiovascular Surgery* 136 (4): 1098–1099. doi:10.1016/j.jtcvs.2008.03.055.
- Sodian, R., S. Weber, M. Markert, D. Rassoulain, I. Kaczmarek, T. C. Lueth, B. Reichart, and S. Daebritz. 2007. Stereolithographic models for surgical planning in congenital heart surgery. *The Annals of Thoracic Surgery* 83 (5): 1854–1857. doi:10.1016/j.athoracsur.2006.12.004.
- Songur, C. M., E. Simsek, A. Ozen, S. Kocabeyoglu, and T. A. Donmez. 2014. Long term results comparing mechanical and biological prostheses in the tricuspid valve position: Which valve types are better-mechanical or biological prostheses? *Heart, Lung & Circulation* 23 (12): 1175–1178. doi:10.1016/j.hlc.2014.05.015.
- Steinhoff, G., U. Stock, N. Karim, H. Mertsching, A. Timke, R. R. Meliss, K. Pethig, A. Haverich, and A. Bader. 2000. Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits: *in vivo* restoration of valve tissue. *Circulation* 102 (suppl 3): Iii-50–Iii-55. doi:10.1161/01.CIR.102.suppl_3.III-50.
- Stella, J. A., and M. S. Sacks. 2007. On the biaxial mechanical properties of the layers of the aortic valve leaflet. *Journal of Biomechanical Engineering* 129 (5): 757–766. doi:10.1115/1.2768111.
- Stelzer, P. 2011. The ross procedure: State of the art 2011. *Seminars in Thoracic and Cardiovascular Surgery* 23 (2): 115–123. doi:10.1053/j.semtcvs.2011.07.003.
- Sumikura, H., Y. Nakayama, K. Ohnuma, Y. Takewa, and E. Tatsumi. 2014. In vitro evaluation of a novel autologous aortic valve (biovalve) with a pulsatile circulation circuit. *Artificial Organs* 38 (4): 282–289. doi:10.1111/aor.12173.
- Sumikura, H., Y. Nakayama, K. Ohnuma, Y. Takewa, and E. Tatsumi. 2016. Development of a stent-biovalve with round-shaped leaflets: In vitro hydrodynamic evaluation for transcatheter pulmonary valve implantation (TPVI). *Journal of Artificial Organs* 19 (4): 357–363. doi:10.1007/s10047-016-0909-6.
- Syedain, Z. H., L. A. Meier, J. M. Reimer, and R. T. Tranquillo. 2013. Tubular heart valves from decellularized engineered tissue. *Annals of Biomedical Engineering* 41 (12): 2645–2654. doi:10.1007/s10439-013-0872-9.
- Syedain, Z., J. Reimer, J. Schmidt, M. Lahti, J. Berry, R. Bianco, and R. T. Tranquillo. 2015. 6-month aortic valve implantation of an off-the-shelf tissue-engineered valve in sheep. *Biomaterials* 73: 175–184. doi:10.1016/j.biomaterials.2015.09.016.
- Takagi, K., S. Fukunaga, A. Nishi, T. Shojima, K. Yoshikawa, H. Hori, H. Akashi, and S. Aoyagi. 2006. In vivo recellularization of plain decellularized xenografts with specific cell characterization in the systemic circulation: Histological and immunohistochemical study. *Artificial Organs* 30 (4): 233–241. doi:10.1111/j.1525-1594.2006.00210.x.
- Taylor, P. A., P. Batten, N. J. Brand, P. S. Thomas, and M. H. Yacoub. 2003. The cardiac valve interstitial cell. *International Journal of Biochemistry & Cell Biology* 35: 113–118.
- Taylor, P. M., S. P. Allen, S. A. Dreger, and M. H. Yacoub. 2002. Human cardiac valve interstitial cells in collagen sponge: A biological three-dimensional matrix for tissue engineering. *The Journal of Heart Valve Disease* 11 (3): 298–307.
- Taylor, P. M., E. Sachlos, S. A. Dreger, A. H. Chester, J. T. Czernuszka, and M. H. Yacoub. 2006. Interaction of human valve interstitial cells with collagen matrices manufactured using rapid prototyping. *Biomaterials* 27 (13): 2733–2737. doi:10.1016/j.biomaterials.2005.12.003.

- Tedder, M. E., J. Liao, B. Weed, C. Stabler, H. Zhang, A. Simionescu, and D. T. Simionescu. 2009. Stabilized collagen scaffolds for heart valve tissue engineering. *Tissue Engineering. Part A* 15 (6): 1257–1268. doi:10.1089/ten.tea.2008.0263.
- Tedder, M. E., A. Simionescu, J. Chen, J. Liao, and D. T. Simionescu. 2011. Assembly and testing of stem cell-seeded layered collagen constructs for heart valve tissue engineering. *Tissue Engineering. Part A* 17 (1–2): 25–36. doi:10.1089/ten.TEA.2010.0138.
- Theodoridis, K., I. Tudorache, A. Calistru, S. Cebotari, T. Meyer, S. Sarikouch, C. Bara, R. Brehm, A. Haverich, and A. Hifiker. 2015. Successful matrix guided tissue regeneration of decellularized pulmonary heart valve allografts in elderly sheep. *Biomaterials* 52: 221–228. doi:10.1016/j.biomaterials.2015.02.023.
- Thubrikar, M., W. C. Piepgrass, J. D. Deck, and S. P. Nolan. 1980. Stresses of natural versus prosthetic aortic valve leaflets in vivo. *The Annals of Thoracic Surgery* 30 (3): 230–239.
- Torre, I. G. de, M. Weber, L. Quintanilla, M. Alonso, S. Jockenhoevel, J. C. R. Cabello, and P. Mela. 2016. Hybrid elastin-like recombinamer-fibrin gels: Physical characterization and in vitro evaluation for cardiovascular tissue engineering applications. *Biomaterials Science*. doi:10.1039/C6BM00300A.
- Tremblay, C., J. Ruel, J.-M. Bourget et al. 2014. A new construction technique for tissue-engineered heart valves using the self-assembly method. *Tissue Engineering Part C: Methods* 20 (14): 905–916. doi:10.1089/ten.TEC.2013.0698.
- Tseng, H., D. S. Puperi, E. J. Kim, S. Ayoub, J. V. Shah, M. L. Cuchiara, J. L. West, and K. J. Grande-Allen. 2014. Anisotropic poly(ethylene glycol)/polycaprolactone hydrogel-fiber composites for heart valve tissue engineering. *Tissue Engineering. Part A* 20 (19–20): 2634–2645. doi:10.1089/ten.TEA.2013.0397.
- Tudorache, I., K. Theodoridis, H. Baraki, S. Sarikouch, C. Bara, T. Meyer, K. Höffler et al. 2016. Decellularized aortic allografts versus pulmonary autografts for aortic valve replacement in the growing sheep model: Haemodynamic and morphological results at 20 months after implantation. *European Journal of Cardio-Thoracic Surgery: Official Journal of the European Association for Cardio-Thoracic Surgery* 49 (4): 1228–1238. doi:10.1093/ejcts/ezv362.
- Vafae, T., D. Thomas, A. Desai, L. M. Jennings, H. Berry, P. Rooney, J. Kearney, J. Fisher, and E. Ingham. 2016. Decellularisation of human donor aortic and pulmonary valved conduits using low concentration SDS. *Journal of Tissue Engineering and Regenerative Medicine*. doi:10.1002/term.2391.
- Vahanian, A., O. Alfieri, F. Andreotti, M. J. Antunes, G. Barón-Esquivias, H. Baumgartner, M. A. Borger et al. 2012. Guidelines on the management of valvular heart disease (version 2012). *European Heart Journal* 33 (19): 2451–2496. doi:10.1093/eurheartj/ehs109.
- VeDepo, M. C., E. E. Buse, R. W. Quinn, T. D. Williams, M. S. Detamore, R. A. Hopkins, and G. L. Converse. 2017. Species-specific effects of aortic valve decellularization. *Acta Biomaterialia* 50: 249–258. doi:10.1016/j.actbio.2017.01.008.
- Vesely, I. 1998. The role of elastin in aortic valve mechanics. *Journal of Biomechanics* 31 (2): 115–123.
- Vesely, I., and R. Noseworthy. 1992. Micromechanics of the fibrosa and the ventricularis in aortic valve leaflets. *Journal of Biomechanics* 25 (1): 101–113.
- Voges, I., J. H. Bräsen, A. Entenmann, M. Scheid, J. Scheewe, G. Fischer, C. Hart et al. 2013. Adverse results of a decellularized tissue-engineered pulmonary valve in humans assessed with magnetic resonance imaging. *European Journal of Cardio-Thoracic Surgery* 44 (4): e272–e279. doi:10.1093/ejcts/ezt328.
- Wang, H., B. Sridhar, L. A. Leinwand, and K. S. Anseth. 2013. Characterization of cell sub-populations expressing progenitor cell markers in porcine cardiac valves. *PLoS ONE* 8 (7). doi:10.1371/journal.pone.0069667.

- Weber, B., P. E. Dijkman, J. Scherman, B. Sanders, M. Y. Emmert, J. Grünenfelder, R. Verbeek et al. 2013. Off-the-shelf human decellularized tissue-engineered heart valves in a non-human primate model. *Biomaterials* 34 (30): 7269–7280. doi:10.1016/j.biomaterials.2013.04.059.
- Weber, B., M. Y. Emmert, L. Behr et al. 2012. Prenatally engineered autologous amniotic fluid stem cell-based heart valves in the fetal circulation. *Biomaterials* 33: 4031–4043.
- Weinberg, E. J., P. J. Mack, F. J. Schoen, G. García-Cardena, and M. R. Kaazempur Mofrad. 2010. Hemodynamic environments from opposing sides of human aortic valve leaflets evoke distinct endothelial phenotypes in vitro. *Cardiovascular Engineering (Dordrecht, Netherlands)* 10 (1): 5–11. doi:10.1007/s10558-009-9089-9.
- Welke, K. F., Y. Wu, G. L. Grunkemeier, A. Ahmad, and A. Starr. 2011. Long-term results after carpentier-edwards pericardial aortic valve implantation, with attention to the impact of age. *The Heart Surgery Forum* 14 (3): E160–E165. doi:10.1532/HSF98.20101140.
- Wu, S., B. Duan, P. Liu, C. Zhang, X. Qin, and J. T. Butcher. 2016a. Fabrication of aligned nanofiber polymer yarn networks for anisotropic soft tissue scaffolds. *ACS Applied Materials & Interfaces* 8 (26): 16950–16960. doi:10.1021/acsami.6b05199.
- Wu, S., B. Duan, X. Qin, and J. T. Butcher. 2017. Living nano-micro fibrous woven fabric/hydrogel composite scaffolds for heart valve engineering. *Acta Biomaterialia* 51: 89–100. doi:10.1016/j.actbio.2017.01.051.
- Wu, S. H., and X. H. Qin. 2013. Uniaxially aligned polyacrylonitrile nanofiber yarns prepared by a novel modified electrospinning method. *Materials Letters* 106: 204–207. doi:10.1016/j.matlet.2013.05.010.
- Wu, S., X. Qin, and M. Li. 2014. The structure and properties of cellulose acetate materials: A comparative study on electrospun membranes and casted films. *Journal of Industrial Textiles* 44 (1): 85–98. doi:10.1177/1528083713477443.
- Wu, S., Y. Zhang, P. Liu, and X. Qin. 2016b. Polyacrylonitrile nanofiber yarns and fabrics produced using a novel electrospinning method combined with traditional textile techniques. *Textile Research Journal* 86 (16): 1716–1727. doi:10.1177/0040517515603808.
- Yacoub, M. H., and J. J. M. Takkenberg. 2005. Will heart valve tissue engineering change the world? *Nature Clinical Practice Cardiovascular Medicine* 2 (2): 60–61. doi:10.1038/npcardio0112.
- Ye, Q., G. Zünd, P. Benedikt, S. Jockenhoevel, S. P. Hoerstrup, S. Sakyama, J. A. Hubbell, and M. Turina. 2000. Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering. *European Journal of Cardio-Thoracic Surgery* 17 (5): 587–591. doi:10.1016/S1010-7940(00)00373-0.
- Yip, C. Y. Y., J. H. Chen, R. Zhao, and C. A. Simmons. 2009. Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. *Arteriosclerosis, Thrombosis, and Vascular Biology* 29 (6): 936–942. doi:10.1161/ATVBAHA.108.182394.
- Zafar, F., R. B. Hinton, R. A. Moore, R. Scott Baker, Roosevelt Bryant III, Daria A. Narmoneva, Michael D. Taylor, and David L. Morales. 2015. Physiological growth, remodeling potential, and preserved function of a novel bioprosthetic tricuspid valve: Tubular bioprosthesis made of small intestinal submucosa-derived extracellular matrix. *Journal of the American College of Cardiology* 66 (8): 877–888. doi:10.1016/j.jacc.2015.06.1091.



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Chapter 11 3D bioprinting of cardiac muscle tissue

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11.1 Introduction

Ischemic heart disease kills millions of people across the world every year and has contributed to >13% of total global deaths in 2012 [1]. The vast majority of these heart diseases are slow, degenerative conditions that eventually lead to heart failure over a period of months to years. Thus, there is time and opportunity to intervene in the disease process and repair the damage. The heart, however, does not have the ability to regenerate or undergo repair that is sufficient to overcome myocardial infarction (MI) or other types of cardiomyopathy. A substantial number of therapies have been developed to mitigate the disease processes, but these efforts generally only slow disease progression.

Over the past decade, advances in cardiac tissue engineering have demonstrated that it is possible to generate myocardium-like tissue constructs with structural

and functional properties that start to recapitulate native muscle. However, the contractile properties and size have remained limited, demonstrating that significant improvements are still needed. For example, neonatal rat cardiomyocytes have been used to engineer cardiac muscle with electromechanical properties that are comparable to native tissue [2–6] and implanted back into rat heart to augment contractile function. Similar techniques have enabled the engineering of myocardium from embryonic stem cell (ESC)-derived cardiomyocytes as well [7–9]. However, these constructs are often of low density and not well aligned. Further, vascularization is only achieved when constructs are implanted *in vivo* and/or cocultured with endothelial cells in specific conditions. Thus, organizing cardiomyocytes, and potentially endothelial cells, to approximate or mimic the 3D structure of stereotypical myocardium is still a major and unresolved challenge.

For engineering human cardiac muscle tissue, pluripotent stem cells (PSCs) now enable the derivation of large numbers of human cardiomyocytes from renewable cell sources. Recent advances have demonstrated the differentiation of cardiomyocytes from both ESCs [10–12] and induced pluripotent stem cells (iPSCs) [13,14], as well as direct reprogramming from cardiac fibroblasts [15,16]. Further, research in organoids has demonstrated that pluripotent and multipotent stem cells have a remarkable ability to recapitulate developmental morphogenetic programs [17] and to self-assemble into complex tissue structures including optic cups [18,19], primitive brain tissue [20,21], intestinal tissue [22], and teeth [23]. However, despite these impressive examples, these tissues hit a developmental roadblock as the diffusion limit of oxygen is reached. At least in part, it is the failure of vascularization to form that limits further growth and development. This demonstrates that though differentiation and early morphogenesis appear to be recapitulated *in vitro*, vascularization remains a barrier that current systems have not been able to overcome beyond small, lab-on-chip systems [24,25]. New, innovative solutions are needed to engineer human cardiac muscle tissue with dense vascularization and at large volumes.

This is where 3D bioprinting is uniquely positioned because it offers the potential to fabricate cardiac tissues with control of multiscale architecture. However, being able to print tissue that can contract, in and of itself, is not enough to build functional cardiac muscle. The heart is a complex organ with a unique, multiscale architecture composed of a wide range of cell types with specialized functions. Although it may not be necessary to recreate all of this complexity to build functional heart muscle, certain aspects such as adequate force generation and nutrient supply to the muscle cells are key requisites. In this chapter, we will first discuss the basic design requirements for engineering 3D heart muscle tissue, with a focus on how the embryonic and adult hearts are organized and the design principles that can be developed from this. Second, we will review the types of bioinks that can be used for 3D bioprinting cardiac muscle and the specific properties that are needed. Third, we will discuss some of the limited but important examples

of 3D bioprinting heart muscle in the literature. And finally, we will consider the steps that need to be taken toward engineering a whole heart at the organ scale with a focus on medical imaging for patient-specific constructs, advances in 3D bioprinting needed for large volumetric tissues, and the bioreactor systems that will be needed to ultimately maintain and mature these 3D-bioprinted organs so that they can be implanted in patients.

11.2 Design criteria for engineering cardiac muscle tissue

The heart is a well-studied organ, with a range of vertebrate animal models that have provided detailed information on how the heart is structured in 3D [26,27]. With the emergence of 3D bioprinting, it is now possible to draw basic engineering design principles from these studies and apply them, specifically in terms of architecture, cellular and extracellular matrix (ECM) composition, and mechanical properties. For example, the adult human heart provides a template for designing large volume contractile tissues capable of generating large forces, whereas developmental models of the chicken and rodent heart provide an insight into how to dynamically organize cells and ECM proteins and transition from a simple heart tube into a stereotypic four-chambered heart. Here we first focus on the adult heart and identify specific design principles that can be applied to 3D-bioprinting adult-stage cardiac muscle tissues. Then we describe the design principles from the dynamic environment of the embryonic heart, specifically how we can use this to potentially engineer complex 3D organ structure by recapitulating aspects of cardiac morphogenesis.

11.2.1 Lessons from the adult heart

The adult mammalian heart has a hierarchical architecture organized across multiple biological length scales. When carefully dissected, it has been shown that the heart can be unraveled into a tissue syncytium where the muscle cells form aligned sheets that wrap around the ventricles in distinct layers (Figure 11.1a) [28]. This structural anisotropy is multiscale; at the cellular scale, the cylindrically shaped cardiomyocytes are electrically and mechanically coupled end-to-end at the intercalated disks (Figure 11.1b) and are bundled into myocardial fibers by surrounding ECM fibrils that run in parallel to the longitudinal axis of the cells (Figure 11.1c) [29]. These well-aligned myocardial fibers are arranged into laminar structures that are four to six cells thick, separated from adjacent laminas by additional ECM mesh. The myofibers in their laminar arrangement wrap helically around the heart in an overlapping fashion, and the transmural myofiber orientation rotates for about 120° traversing from the epicardium to the endocardium

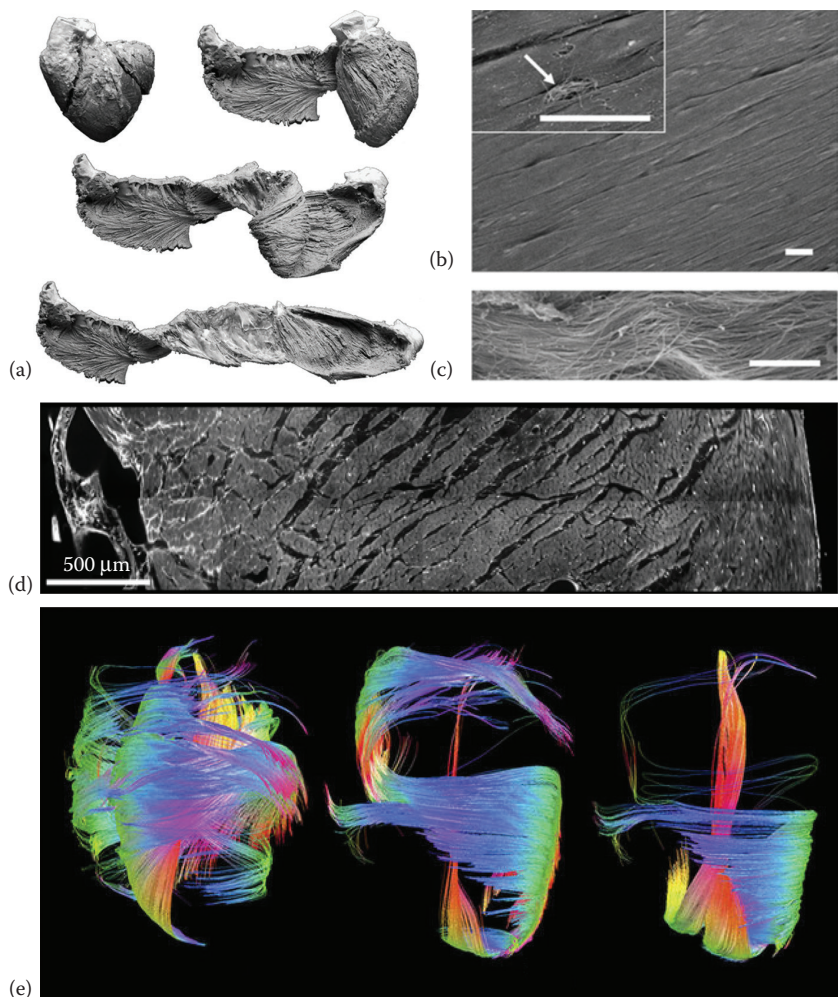


Figure 11.1 Multiscale hierarchical architecture of the heart. (a) Photographs of a bovine heart during dissection as it is unraveled to form a continuous myocardial band. (b) Cardiomyocytes in the heart align to form myofibers, surrounded by (c) a fibrous extracellular matrix that guides cellular alignment and function. (d) Transverse section of an adult heart shows that myofiber alignment orientation changes from the endocardium (left) to the epicardium (right). (e) Diffusion Tensor MRI images at various view angles reveal an overall helical arrangement of the myocardial fibers in the geometry of the heart. (Adapted from Kocica, M.J. et al., *Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu* 10, 52–60, 2007; Kim, D.H. et al. *Proc. Natl. Acad. Sci.*, 107, 565–570, 2009; Young, A.A. et al., *J Microsc.*, 192, 139–150, 1998; Teh, I. et al., *Sci Rep.*, 6, 30573, 2016 for figure panels a, b–c, d, and e respectively. With permission.)

(Figure 11.1d, e) [30]. This helical laminar arrangement of cells and myofibers is necessary for translating uniaxial shortening of sarcomeres within individual cardiomyocytes into efficient 3D ventricular contraction during systolic contraction. Recreating this anisotropic, multiscale architecture is crucial if cardiomyocytes are to be used as the basic contractile unit for an engineered heart that can efficiently pump.

In addition to architectural considerations for constructing a functional cardiac tissue, cellular and connective tissue compositions are also important design elements. The adult mammalian heart is composed primarily of cardiomyocytes, fibroblasts, endothelial cells, and perivascular cells. Although cardiomyocytes occupy more than 70% of the heart volume, histological analysis and flow cytometry have shown that cardiomyocytes are only about 30%–40% of the total cell population [31]. The remaining 60%–70% of the cell population are noncardiomyocytes, suggesting there may be critical roles for these supporting cells in maintaining cardiac homeostasis, generating ECM, ensuring mechanical continuity, and creating vasculature. Unfortunately, the exact proportions of endothelial cells and fibroblasts have varied depending on the methods applied for population analysis and the animal models used [32–34]. Nevertheless, balancing cellular compositions of cardiomyocytes and their supporting cells is essential for maximizing both contractile stress and tissue survival in an engineered cardiac tissue. Just as different cell types play diverse functional roles in the heart, a wide array of cardiac ECM proteins provides distinct mechanical and chemical roles. For example, collagen type I is important for mechanical strength and elastin is important for elasticity of cardiac muscle tissue. Further, glycosaminoglycans (GAGS) are heavily involved in tissue organization, repair, and homeostasis, and ECM proteoglycans bind growth factors and other matrix molecules to protect the heart against injury and diseases [35,36]. Inclusion of multiple ECM components is likely a necessity for constructing large cardiac muscle tissue with physiologic function, though we still need more information on the localization and quantity of each component within the cardiac ECM.

11.2.2 Lessons from the embryonic heart

Embryogenesis is the only time during human development where new cardiac muscle tissue is formed and is characterized by rapid growth and vascularization, suggesting that there are unique cellular populations, ECM composition and structure, growth factors, and mechanical forces at play [37–40]. This is in stark contrast to the adult heart, which is characterized by minimal regenerative capacity, <1% annually [41], due to terminally differentiated cardiomyocytes and adult resident cardiac stem cells that are unable to repair the heart during most cardiac disease states. Based on these facts, embryonic development provides examples of how myocardium forms and vascularizes, from which we can

potentially identify the factors that are necessary to recapitulate this process in a tissue-engineered system.

3D bioprinting has the ability to fabricate very complex structures, making it possible to potentially engineer cardiac muscle tissue at various stages of development from the linear heart tube to four-chambered heart (Figure 11.2a). In the gastrulating vertebrate embryo, cardiac progenitor cells (CPCs) can be first found as an epithelial population of cells that reside in a crescent-shaped region known as the cardiac crescent (Figure 11.2b) [42]. These cells converge toward the center of the crescent to form a linear heart tube (Figure 11.2c) with early regional specification for different parts of the heart. These precursor regions are brought into an adult-like spatial arrangement as the transient heart tube begins to loop and the bottom of the heart tube spirals cranially (Figure 11.2d). The heart then starts to resemble the adult heart when several muscular septa form to create chambers

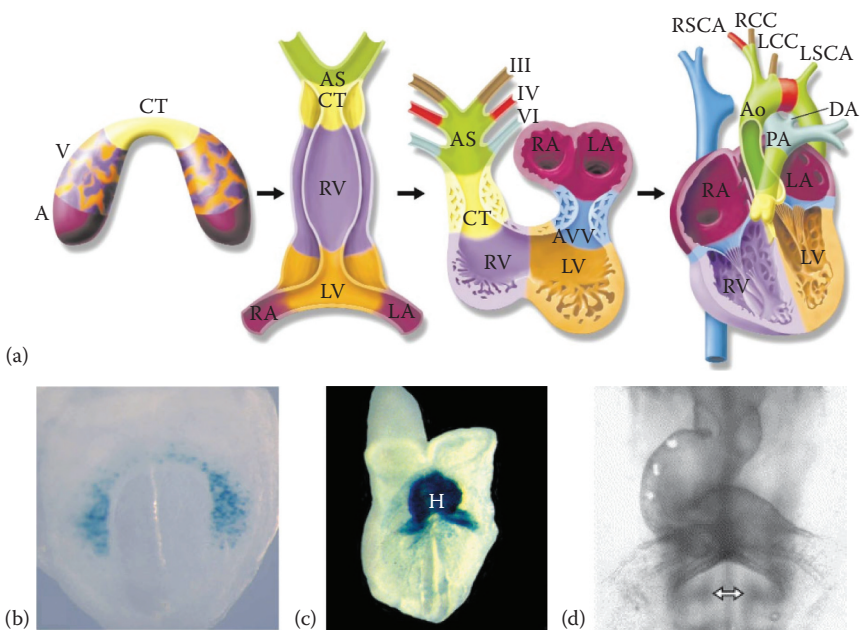


Figure 11.2 Embryonic heart development and structural features. (a) Schematic showing the early stages of embryonic development that includes, from left to right, cardiac crescent formation, heart tube development, heart tube looping, and eventual septation and maturation. Regional patterning begins in the cardiac crescent and can be seen in the heart tube with the corresponding colors. (b) Cells in the cardiac crescent, stained in mice, migrate toward the midline to form the (c) heart tube, which then (d) loops to form an intermediate cardiac structure. (Continued)



Figure 11.2 (Continued) Embryonic heart development and structural features. (e) The embryonic ventricles are primarily lined with muscular protrusions called trabeculations (left), which begin to compact to form a thick myocardium (middle) until cardiac development is complete (right). A, atrium; Ao, aorta; CT, conotruncal; DA, ductus arteriosus; H, heart tube; LA, left atrium; LCC, left common carotid; LSCA, left subclavian artery; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RCC, right common carotid; RSCA, right subclavian artery; RV, right ventricle; and V, ventricle. (Adapted from Srivastava D and Olson E, *Nature*, 407, 221–226, 2000; Ryckebusch, L et al. *Proc. Natl. Acad. Sci.*, 105, 2913–2918, 2008; Chu, P-H et al., *Mech. Dev.* 95:259–265; Voronov, D.A. et al., *Dev. Biol.* 272, 339–350, 2004; Mohun, T.J. and Weninger W.J. *Curr. Opin. Genet. Dev.*, 21, 573–578, 2011 for figure panels a, b, c, d, and e, respectively. With permission.)

of the heart and to separate the outflow tract into the pulmonary trunk and aorta, whereas the valves complete their development from valvular cell precursors [43]. During this time, an extensive trabecular network of muscular protrusions develops in the ventricles, giving the heart a high surface area to volume ratio so that muscle mass can increase without the need for vascularization of the heart itself (Figure 11.2e). As the coronary vascular system develops, the trabecular structures begin to compact and thicken to form the myocardium, and the helical arrangement of myofibers described in the adult heart starts to take form.

Similar to all developmental processes, the series of events described earlier are dynamic and are executed with precise coordination of spatial and temporal patterning [44]. Our ability to pattern CPCs and their extracellular environment in both time and space will be the key to replicating this process *in vitro* in engineered cardiac tissues. The identification and definition of cellular, ECM, and molecular building blocks is of equal importance. A growing body of research has identified two distinct progenitor cell populations residing in the morphoregulatory cardiac crescent prior to formation of the heart tube: the first population is

responsible for heart tube formation and eventually fated to become the right ventricle and the outflow tract (primary heart field) [45], whereas the second population migrates toward the heart tube to form the left ventricle and atrioventricular canal (secondary heart field) [46,47]. Identifying and understanding the behavior of CPCs are important because we can drive these cells to differentiate into multiple cardiac subtypes and thus can leverage the progenitor cells' own biological machinery to achieve cellular and structural maturity in an engineered cardiac tissue.

In addition to the cells, the ECM is also constantly changing during cardiac development to support cellular and organ needs. For example, it is known that fibronectin is expressed heavily during embryonic development to help guide CPC migration during heart tube formation [48]. However, fibronectin is only a small percentage of the adult heart ECM, which at maturity contains a substantial proportion of collagen type I for structural support [49]. Understanding the spatio-temporal composition of a rapidly changing ECM that also includes hyaluronic acid, fibrillin, elastin, and proteoglycans will be useful for guiding cardiac tissue maturation. And just as important as the cells and ECM, molecular gradients of signaling factors such as BMP [50], FGF [51], and Wnt [52,53] are also found to vary with the embryonic stage and location within the embryo, allowing cells to adopt the correct role during each stage of cardiac morphogenesis. As cardiomyocytes derived from iPSCs *in vitro* are typically immature and resemble embryonic cardiomyocytes in their phenotype and genetic program, it is reasonable to propose that use of these cardiomyocytes in engineered tissues must be carefully coupled with a matching embryonic-like scaffold environment. Intricate fabrication and culture platforms will also need to be developed to introduce spatial and temporal cues of growth factors to guide tissue development. Taken together, it is clear that the temporal and spatial patterning events during cardiac morphogenesis are important, but more information is needed on how this process evolves in 3D over time. Looking forward, fully defining the developmental blueprint may enable 3D tissue fabrication technologies that will improve our ability to create biologically complex and mature cardiac tissues.

11.3 Bioinks designed for cardiac tissue printing

The basic building blocks of the human heart consists of ECM proteins such as hyaluronic acid, proteoglycans, fibronectin, fibrillin, elastin, and collagen as well as various cell types including cardiomyocytes, endothelial cells, and fibroblasts. Although it would be ideal if we could precisely arrange each of these cardiac building blocks in anatomically accurate locations, current technology limits the resolution and the number of distinct materials that can be 3D bioprinted. Further, many of these native ECM proteins and cells are difficult and/or expensive to

obtain in quantities that are needed for building large volumetric tissues. For these reasons, bioinks for 3D printing need to address a number of key factors to enable bottom-up fabrication of the heart.

Several design considerations must be made when selecting an ideal bioink that is compatible with current 3D-bioprinting technologies. First, depending on the printing technique used, bioink rheology must be controlled to ensure accurate deposition. For example, inkjet [54] and laser-based approaches [55] require the use of low-viscosity fluids for repeatable droplet formation and ejection, whereas extrusion-based printers are capable of handling bioinks with a wider range of rheological properties [56] including viscoelastic materials, shear-thinning fluids, and yield-stress fluids. Once deposited, the bioink needs to provide structural integrity to maintain the printed geometry. Following traditional fused deposition modeling (FDM) principles, most bioprinting techniques rely on a material that can be accurately extruded as a liquid and can quickly transition to a solid once deposited to achieve high-print fidelity. This transition, or gelling behavior, can be ionic in the case of calcium-cross-linked alginate, physical for temperature and pH-sensitive collagen type I or gelatin, enzymatic for fibrin gels formed from thrombin cleavage of fibrinogen, or photochemical for methacrylated gelatin or hyaluronic acid. Various printer configurations can be designed to rapidly cross-link these materials so that they remain stable once deposited [57]. Importantly, for bioinks, both cells and the biomaterials used are critical interacting components that must be considered when developing materials for bioprinting cardiac tissues. For cell-laden bioinks, the recent development of human iPSCs provides a patient-specific source for pluripotent cells that is capable of generating large numbers of not only cardiomyocytes but also the supporting cell types in the adult heart. Multiple ECM-based hydrogels such as collagen type I, Matrigel, and fibrin have been demonstrated to support tissue development in engineered cardiac tissues cast from molds, representing potential materials for bioink formulations [58].

To satisfy the design requirements for cardiac tissue in terms of structure, ECM composition, and cell type, it is unlikely that one *ideal bioink* exists for 3D-bioprinting cardiac muscle. Instead, it is necessary to design multiple bioink formulations and print them in combination using a 3D bioprinter capable of dispensing multiple materials. Different bioinks with specific structural, biological, and chemical roles will enable us to create architecturally and functionally complex cardiac tissues. Although nonnative structural materials such as alginate [59,60] and polyethylene glycol [61] have been used in constructing 3D cardiac tissues, these materials lack biological activity and do not support the level of cardiac tissue development observed with native hydrogels such as collagen type I and fibrin, which can be compacted into dense muscle tissue. Addition of Matrigel, an ECM derivative from Engelbrecht-Holm-Swarm mouse tumors that contain basement membrane proteins such as collagen type IV and laminin, demonstrates

that inclusion of additional native ECM components can further enhance muscle formation in terms of cardiomyocyte alignment and increased contractile stress [62,63]. Following this paradigm of using a hydrogel that more closely resembles the adult heart ECM as a bioink, hydrogels have been derived directly from decellularized cardiac ECM and shown to be printable and support cardiomyocyte survival [64]. It is crucial to remember that the heart is not constructed from a homogenous mixture of cells and ECM but instead has well-defined compartments for myofibers, dense acellular ECM fibrils surrounding these myofibers, and vasculature lumens lined with a basement membrane that supports a layer of endothelial cells. For example, a complete cellular cardiomyocyte bioink deposited along with a cardiac ECM mixture bioink can allow us to begin to architect the honeycomb-like ECM compartments for myofibers [65] and to better recreate both native cellular and ECM fibril density, both of which has been shown to improve contractile stress in engineered cardiac tissues. Similar cellular and acellular bioink strategies can be followed for constructing cardiac vasculatures with subsequent bioink layers of endothelial cells, surrounded by basement membrane proteins, and lined with a thick outer layer of smooth muscle cells and connective tissue proteins. However, to date most cardiac tissue engineering has used scaffold materials, essentially bioinks, that are micromolded or otherwise casted to generate 3D scaffold muscle formation [66]. As more researchers work to 3D-bioprint cardiac muscle tissue, it is anticipated that the properties of bioinks will continue to improve.

11.4 Current progress in 3D bioprinting of cardiac muscle constructs

Current progress in the 3D bioprinting of cardiac muscle has primarily focused on the development of contractile tissues that can be used near-term as *in vitro* models for drug development, with the long-term goal to be implanted *in vivo* into an animal model of MI to improve cardiac function. Although various additive manufacturing technologies have been used to fabricate these constructs, most approaches follow an established tissue engineering paradigm, utilizing a bio-compatible hydrogel as a scaffold to encapsulate cells in 3D [67]. For example, sodium alginate (Figure 11.3a and b) [68], gelatin mixed with hyaluronic acid [69], and methacrylated gelatin mixed with sodium alginate [70] have all been found to preserve cardiomyocyte viability after printing and to maintain printed geometry. Gaetani et al. demonstrated that human cardiac progenitor cells (hCPCs) printed in alginate increased TroponinT gene expression over 7 days, though lacked organized sarcomeres indicative of cardiac maturation. Follow-up work by the same group showed that using a bioink composed of gelatin mixed with hyaluronic acid directed hCPCs toward a more mature phenotype. Similarly, Zhang et al.

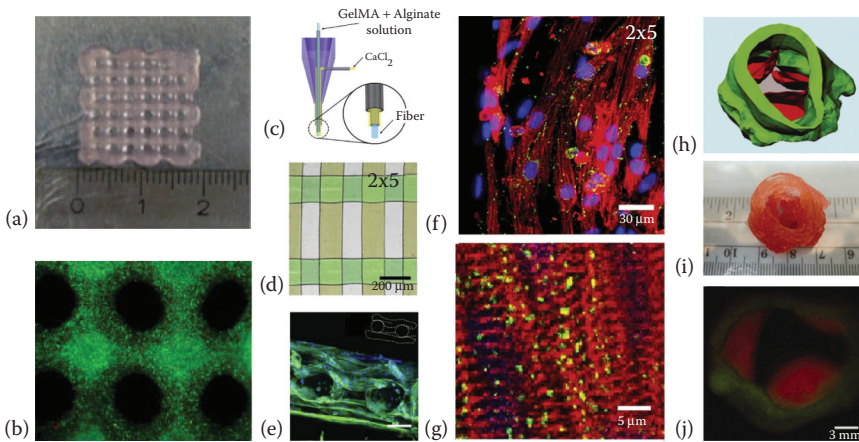


Figure 11.3 3D bioprinting of cardiac tissues. (a) Extrusion-based printing of an alginate construct shows (b) high cell viability of embedded cardiomyocytes. (c) Using a coaxial extrusion system to simultaneously deposit a bioink and a cross-linking solution (CaCl₂), (d) layers of orthogonal lines can be printed, (e) human umbilical vein endothelial cells (HUVECs) mixed into the bioink migrate to the surface of the printed strands, and cardiomyocytes seeded onto the construct display (f) good alignment and (g) organized sarcomeres. A model of a porcine valve can also be generated from (h) micro-CT data, (i) printed at good fidelity with (j) two different cell types on a multimaterial extrusion-based system. (Adapted from Gaetani, R. et al., *Biomaterials* 33(6):1782–1790, 2012; Zhang, Y.S. et al., *Biomaterials* 110, 45–59, 2016; Duan, B. et al., *J Biomed Mater Res A*, 101A, 1255–1264, 2013 for figure panels a–b, c–g and, h–j, respectively. With permission.)

used methacrylated gelatin mixed with alginate to 3D bioprint a construct composed of rat neonatal cardiomyocytes and endothelial cells with contractile activity (Figure 11.3c–g) [71]. The addition of gold nanorods to this bioink produced improved cellular organization and contraction synchrony, demonstrating that bioink design is important for improving tissue function [71]. Multimaterial systems also allow for additional engineered complexity, as demonstrated in a valve patterned with two different cell types (Figure 11.3h–j) [72]. These studies are important because they show that extrusion-based 3D-bioprinting technology is capable of depositing various cell-laden gelling materials without sacrificing cellular viability and activity.

Despite the progress that has been made in 3D bioprinting of cardiac muscle using extrusion-based approaches, geometries have been primarily limited to orthogonal layers of evenly spaced extruded filaments assembled layer-by-layer. These prints are typically square-lattice structures and are relatively simple compared

to native heart architecture. As demonstrated in [Figure 11.1](#), the architecture of the heart is multiscale and anisotropic in 3D space, and this structure is critical to cardiac function at the tissue and organ scales. A variety of methods have been developed to improve print fidelity and geometric complexity and can be applied to 3D bioprinting of cardiac tissues, including dual cross-linking systems with tunable mechanical properties, extrusion of semigelled materials, and laser-induced forward transfer of cells onto cell-adhesive substrates [73]. To create complex structures using soft materials, a number of 3D-bioprinting methods have been developed. For example, printing sacrificial bioinks in combination with the primary bioinks intended to form the tissue construct enables the creation of perfusable lumens [74,75], and careful design of composite and synthetic bioink properties can lead to higher fidelity prints [76,77]. Also, shape-morphing structures that change over time, also referred to as 4D printing, have the potential to form complex structures from simpler structures that are easier to print [78]. Finally, reversible embedding of ECM hydrogel and other biomaterials in support baths, which will be elaborated later in this chapter, allows potentially unconstrained 3D construction with soft materials [79,80]. In all, improving both bioink properties and printing techniques is necessary for 3D printing to become a feasible technology for creating anatomically and functionally accurate cardiac tissues.

11.5 Toward whole heart engineering

11.5.1 Medical imaging to generate patient-specific heart models

A key advantage of 3D bioprinting is the potential to use medical imaging data to generate patient-specific constructs. This ability to produce accurate 3D digital models of patient hearts is already a standard practice as magnetic resonance imaging (MRI) and computed tomography (CT) are widely used in the clinic for whole heart imaging to diagnose a range of cardiac diseases from MI to valve disease [81–83]. These 3D digital models are routinely 3D printed using plastics to generate physical models used for surgical planning and diagnostic purposes, resulting in decreased surgery time and increased success rates [84,85]. Thus the process of imaging the heart, segmenting the image into a digital model, and then 3D printing a heart replica is already well established [86]. The challenge then is printing living tissue and extracting enough data from the clinical imaging data to engineer a heart that has appropriate structure and function. For example, the heart is the most densely vascularized organ in the body with capillary-to-capillary spacing of approximately 20 μm [87]. This is beyond the resolution of most clinical MRI and CT systems, which have voxel sizes in the range of 1 mm [88]. Although higher resolution micro-CT and higher Tesla MRI systems exist, they

are not routinely used with human patients. However, there are types of imaging that can be performed to provide additional structural information.

One of the most promising technologies for providing information on muscle orientation in the heart is diffusion tensor MRI (DTMRI). DTMRI works by being able to determine the direction of water diffusion within living tissue. As water molecules diffuse faster in the direction of the aligned cardiomyocytes, DTMRI can provide information on the orientation of cardiac muscle tissue in 3D space (Figure 11.4a) [89,90]. As discussed previously, this variable alignment of muscle tissue within the heart wall is important for the contractile behavior of the ventricle and hence is important to maximize ejection fraction (Figure 11.4b). Although researchers have yet to 3D bioprint a beating heart, it is anticipated that this aligned architecture will be a critical feature to replicate. Importantly, DTMRI has the potential to be performed in the clinic [91], providing a realistic pathway to generate patient-specific 3D digital heart models that include overall shape and muscle organization. Now there are limits to CT, MRI, and DTMRI, and none have the resolution to identify individual vessels within the microvasculature. However, it is well established that the capillaries generally run in parallel to the cardiomyocytes, suggesting that the DTMRI image could be used to also determine the architecture of the microvasculature using computational models designed to approximate this structure [92]. To date, CT, MRI, and DTMRI have not been used for the design of a bioprinted heart, but these clinical imaging modalities can provide important details on anatomical structure that in combination with computational tools should provide a path forward.

11.5.2 3D-bioprinting approaches at the organ scale

Bioprinting a heart, or for that matter any complex organ, is a significant challenge and beyond the capabilities of current printing platforms. However, it is clear that there are a number of key issues that need to be addressed to move forward, and realistic pathways are identified to do so. First, the 3D-bioprinting technology used needs to have the potential to recreate the complexity of a human organ such as the heart. Although there are many 3D-bioprinting techniques that may work at the tissue scale, organs are typically much larger and have complex, multicellular, and multimaterial structures (Figures 11.1 and 11.2). This by definition requires a multiple print head system (Figure 11.4c) or a single print head that is capable of changing materials that it is depositing. For this reason, extrusion- and inkjet-based systems that can have multiple nozzles are attractive options [93]. In contrast, laser- and digital light projection (DLP)-based stereolithography (SLA) are not ideal because they typically use a single material with or without a single cell composition. Although it is technically possible to change the photopolymer during an SLA print, this is a complex and time-consuming process. Second, the 3D-bioprinting process needs to be able to deposit soft

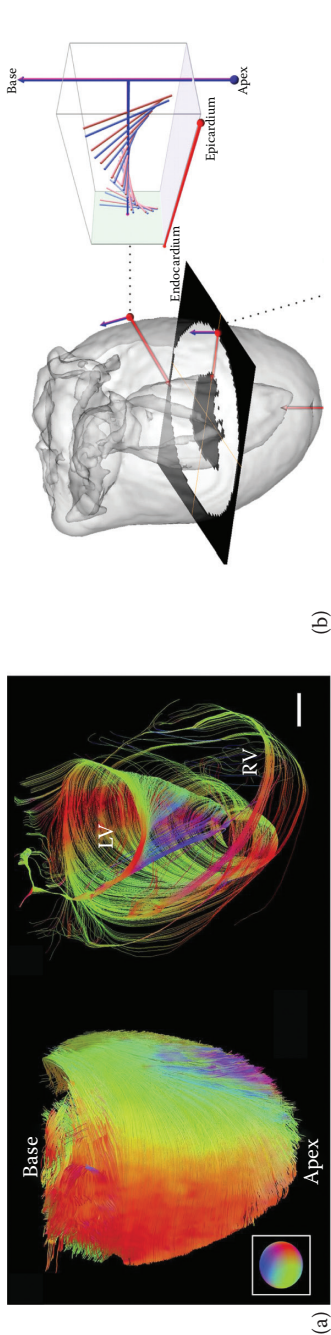


Figure 11.4 Multiple technologies are needed to achieve 3D bioprinting of human hearts using patient-specific anatomic structure, tissue organization, and function. (a) DTMRI tracks water diffusion along the myocardial fibers to give an insight into the helical arrangement of the myocardial fibers and its change in orientation throughout the heart walls. (Continued)

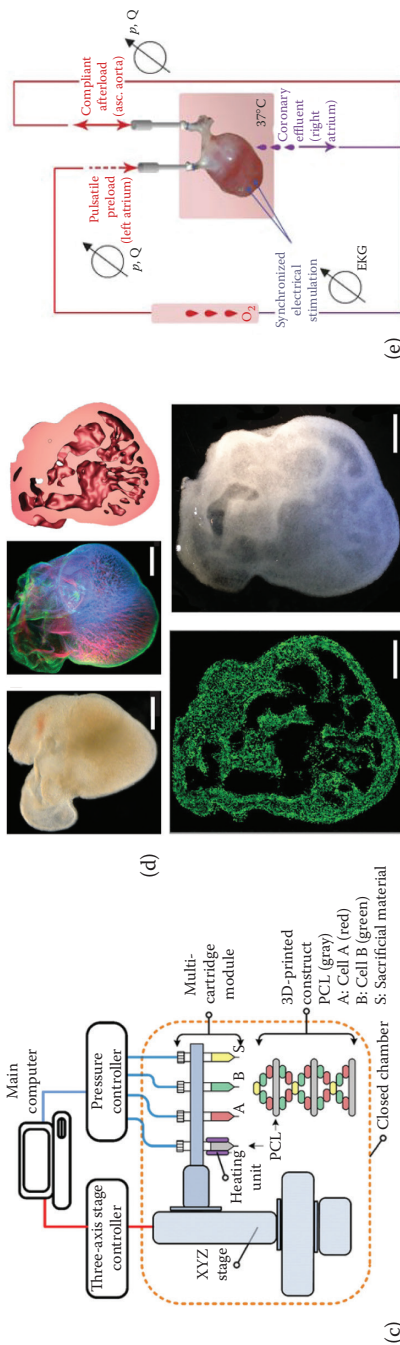


Figure 11.4 (Continued) Multiple technologies are needed to achieve 3D bioprinting of human hearts using patient-specific anatomic structure, tissue organization, and function. (c) A schematic of a multimaterial printer that is capable of depositing materials with distinct structural and functional roles in the final construct. (d) Images showing how the FRESH 3D-bioprinting process can be used to fabricate a whole heart scaffold by fluorescent antibody staining of an embryonic chicken heart that is converted to a printable CAD model, printed using fluorescent alginate and then imaged for print fidelity. (e) Perfusion setup for a decellularized heart is an example for similar bioreactor platforms for maturing a 3D-printed heart. (Adapted from Taylor, E.N. et al., *J Am Heart Assoc* 5, e002836, 2016; Savadijev, P. et al., *Proc. Natl Acad. Sci. USA*, 109, 20166, 2012; Kang, H-W. et al., *Nat. Biotechnol.* 34, 312–319, 2016; Hinton, T.J. et al., *Sci. Adv.*, 1, 2015; Ott, H.C. et al., *Nat. Med.*, 14, 213–221, 2008 for figure panels a, b, c, d, and e, respectively. With permission.)

biological materials in large volumetric structures and with high fidelity. The 3D bioprinting of soft hydrogels, including cell-laden hydrogels, requires the use of support materials that can provide temporary structure. One approach to do this is to print a rigid material such as PCL in a 3D lattice structure and then to print a hydrogel within the open spaces. This has been done by a number of researchers including the printing of muscle constructs that can be implanted *in vivo* [64,94]. Similar work has used Pluronics F127 and carbohydrate glass as sacrificial materials that can be dissolved later to create void space within which hydrogel scaffolds are later cast or coprinted [75,95,96]. More recently, researchers have developed new embedded printing approaches where hydrogels and other soft biomaterials can be 3D bioprinted within a yield stress, gel-based support bath, and then later removed [79,80,97]. Of these, freeform reversible embedding of suspended hydrogels (FRESH) printing by Hinton et al. has demonstrated the ability to 3D-bioprint hydrogel scaffolds at the organ scale, based on architecture of the embryonic chick heart (Figure 11.4d) [79]. Printing cellularized constructs using FRESH that have the appropriate cardiac cell types is a potential approach to organ-scale fabrication. However, this highlights the third challenge, which is obtaining the correct cell types and at sufficient quantity to fabricate volumetric tissues. The heart is composed of a large number of different cell types, including cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells, Purkinje cells, nodal cells, and neurons, and each of these is further divided into subtypes that vary across different regions and structures within the heart [98]. There are also billions of cells within the heart, and generating this number of new cells is a major, unresolved challenge facing the entire 3D-bioprinting and tissue engineering fields [99]. Although organ-scale printing of a functional heart construct is yet to be achieved, important progress is being made on all of the required components, and it is anticipated that multimaterial, multicellular 3D-bioprinted heart constructs will be achieved in the near future.

11.5.3 Development of bioreactor systems for organ maintenance and maturation

Unlike standard 3D printing where the printed part can typically be used with minimal post-processing, 3D bioprinting requires substantial post-processing to generate a functional organ. Whether printing cells directly and/or seeding cells after printing, the cells start out in suspension in a spherical morphology and then must start to spread into, or onto, the surrounding scaffold material. Thus a culture period is required to enable the cells to grow, interconnect, and proliferate so that higher cell densities can be achieved. Specifically, it is important to realize that when 3D-printing cell-laden hydrogels, the cell densities are always much lower when printed than the cell densities within the target tissues such as cardiac muscle. To achieve appropriate densities, the cells must compact the scaffold

over time and/or proliferate while remodeling the surrounding scaffold. At the scale of the whole heart, bioreactor systems need to be developed to perfuse the large volumetric organ and provide adequate fluid flow for nutrient mass transport. One way in which this can be achieved is to use a perfusion system similar to that used for decellularization and recellularization of hearts (Figure 11.4e), which has been demonstrated in rodent hearts and has since been extended to larger porcine and human hearts [100–102]. In addition to perfusion, heart bioreactors need to apply additional stimuli including (1) mechanical loading through pressure or controlled stretch, (2) electrical excitation to control contraction rate, and (3) soluble factors such as T3 hormone to stimulate cardiomyocyte maturation [103,104]. Based on whole-heart perfusion systems and decellularization, the primary components required for bioreactors intended to support the growth and maturation of 3D-bioprinted whole hearts are already established. The next step is to advance 3D-bioprinting technologies to the point of fabricating hearts; maturing them; and then evaluating structure, function, and *in vivo* performance.

References

1. Association AH (2010) Heart disease and stroke statistics–2010 update. A report from the American Heart Association, Dallas, Texas. *Circulation* 121:e46–e215.
2. Alford PW, Feinberg AW, Sheehy SP, and Parker KK (2010) Biohybrid thin films for measuring contractility in engineered cardiovascular muscle. *Biomaterials* 31:3613–3621.
3. Feinberg AW et al. (2007) Muscular thin films for building actuators and powering devices. *Science* 317(5843):1366–1370.
4. Shimizu T et al. (2002) Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circulation Research* 90(3):E40–E48.
5. Baar K et al. (2004) Self-organization of rat cardiac cells into contractile 3-D cardiac tissue. *The FASEB Journal* 18(15):275–277.
6. Zimmermann WH, Melnychenko I, and Eschenhagen T (2004) Engineered heart tissue for regeneration of diseased hearts. *Biomaterials* 25(9):1639–1647.
7. Stevens KR et al. (2009) Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proceedings of the National Academy of Sciences of the United States of America* 106(39):16568–16573.
8. Guo X-M et al. (2006) Creation of engineered cardiac tissue in vitro from mouse embryonic stem cells. *Circulation* 113(18):2229–2237.
9. Domian IJ et al. (2009) Generation of functional ventricular heart muscle from mouse ventricular progenitor cells. *Science* 326(5951):426–429.
10. Braam SR et al. (2008) Improved genetic manipulation of human embryonic stem cells. *Nature Methods* 5(5):389–392.
11. Caspi O et al. (2007) Tissue engineering of vascularized cardiac muscle from human embryonic stem cells. *Circulation Research* 100(2):263–272.
12. Anderson D et al. (2007) Transgenic enrichment of cardiomyocytes from human embryonic stem cells. *Molecular Therapy* 17:2027–2036.
13. Zhang J et al. (2009) Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circulation Research* 104(4):e30–e41.

14. Gai H et al. (2009) Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts. *Cell Biology International* 33(11):1184–1193.
15. Ieda M et al. (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142(3):375–386.
16. Fu J-D and Srivastava D (2015) Direct reprogramming of fibroblasts into cardiomyocytes for cardiac regenerative medicine. *Circulation Journal* 79: 245–254.
17. Sasai Y (2013) Cytosystems dynamics in self-organization of tissue architecture. *Nature* 493(7432):318–326.
18. Eiraku M et al. (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* 472(7341):51–56.
19. Nakano T et al. (2012) Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell* 10(6):771–785.
20. Eiraku M et al. (2008) Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* 3(5):519–532.
21. Lancaster MA et al. (2013) Cerebral organoids model human brain development and microcephaly. *Nature* 501(7467):373–379.
22. Spence JR et al. (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 470(7332):105–109.
23. Ikeda E et al. (2009) Fully functional bioengineered tooth replacement as an organ replacement therapy. *Proceedings of the National Academy of Sciences of the United States of America* 106(32):13475–13480.
24. Baker BM, Trappmann B, Stapleton SC, Toro E, and Chen CS (2013) Microfluidics embedded within extracellular matrix to define vascular architectures and pattern diffusive gradients. *Lab on a Chip* 13(16):3246–3252.
25. Moya M, Alonzo L, and George S (2014) Microfluidic device to culture 3D in vitro human capillary networks. *Biomimetics and Stem Cells, Methods in Molecular Biology*, Eds Vunjak-Novakovic G and Turksen K (Springer New York), Vol 1202, pp. 21–27.
26. Vivien CJ, Hudson JE, and Porrello ER (2016) Evolution, comparative biology and ontogeny of vertebrate heart regeneration. *npj Regenerative Medicine* 1(1): 16012.
27. Jensen B, Wang T, Christoffels VM, and Moorman AFM (2013) Evolution and development of the building plan of the vertebrate heart. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1833(4):783–794.
28. Kocica MJ et al. (2006) The helical ventricular myocardial band: Global, three-dimensional, functional architecture of the ventricular myocardium. *European Journal of Cardio-Thoracic Surgery* 29:S21–S40.
29. Pope AJ, Sands GB, Smaill BH, and LeGrice IJ (2008) Three-dimensional transmural organization of perimysial collagen in the heart. *AJP: Heart and Circulatory Physiology* 295(3):H1243–H1252.
30. Savadjiev P et al. (2012) Heart wall myofibers are arranged in minimal surfaces to optimize organ function. *Proceedings of the National Academy of Sciences of the United States of America* 109(49):20166.
31. Pinto AR et al. (2016) Revisiting cardiac cellular composition novelty and significance. *Circulation Research* 118(3):400–409.
32. Anversa POG, Melissari M, and Loud AV (1980) Stereological measurement of cellular and subcellular hypertrophy and hyperplasia in the papillary muscle of adult rat. *Journal of Molecular and Cellular Cardiology* 12(8):781–795.
33. Tang Y, Nyengaard JR, Andersen JB, Baandrup U, and Gundersen HJG (2009) The application of stereological methods for estimating structural parameters in the human heart. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* 292(10):1630–1647.

34. Bergmann O et al. (2015) Dynamics of cell generation and turnover in the human heart. *Cell* 161(7):1566–1575.
35. Ohtsubo K and Marth JD (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* 126(5):855–867.
36. Aviezer D, Hecht D, Safran M, Eisinger M, David G, and Rayon A (1994) Perlecan, basal lamina proteoglycan, promotes basic growth factor receptor binding, mitogenesis, and angiogenesis. *Cell* 79:1005–1013.
37. Wu SM et al. (2006) Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* 127(6):1137–1150.
38. Patwari P and Lee RT (2008) Mechanical control of tissue morphogenesis. *Circulation Research* 103:234–243.
39. Chien KR, Domian IJ, and Parker KK (2008) Cardiogenesis and the complex biology of regenerative cardiovascular medicine. *Science* 322(5907):1494–1497.
40. Parker KK and Ingber DE (2007) Extracellular matrix, mechanotransduction and structural hierarchies in heart tissue engineering. *Philosophical Transactions of the Royal Society B-Biological Sciences* 362(1484):1267–1279.
41. Bergmann O et al. (2009) Evidence for cardiomyocyte renewal in humans. *Science* 324(5923):98–102.
42. Redkar A, Montgomery M, and Litvin, J (2001) Fate map of early avian cardiac progenitor cells. *Development* 128:2269–2279.
43. Sedmera D and McQuinn T (2008) Embryogenesis of the heart muscle. *Heart Failure Clinics* 4(3):235–245.
44. Harvey RP (2002) Organogenesis: Patterning the vertebrate heart. *Nature Reviews Genetics* 3(7):544.
45. Yutzev KE and Kirby ML (2002) Wherefore heart thou? Embryonic origins of cardiogenic mesoderm. *Developmental Dynamics* 223(3):307–320.
46. Jacobson AG and Sater AK (1988) Features of embryonic induction. *Development* 104:341–359.
47. Mjaatvedt CH et al. (2001) The outflow tract of the heart is recruited from a novel heart-forming field. *Developmental Biology* 238(1):97–109.
48. George EL, Baldwin HS, and Hynes RO (1997) Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells. *Blood* 90(8):3073–3081.
49. Lockhart M, Wirrig E, Phelps A, and Wessels A (2011) Extracellular matrix and heart development. *Birth Defects Research Part A: Clinical and Molecular Teratology* 91(6):535–550.
50. Schultheiss TM, Burch JBE, and Lassar, AB (1997) A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes & Development* 11:451–462.
51. Alsan BH and Schultheiss TM (2002) Regulation of avian cardiogenesis by Fgf8 signaling. *Development* 129:1935–1943.
52. Marvin MJ, Di Rocco G, Gardiner A, Bush SM, and Lassar AB (2001) Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Development* 15:316–327.
53. Schneider VA and Mercola M (2001) Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. *Genes & Development* 15:304–325.
54. Cui X, Boland T, D’Lima DD, and Lotz MK (2012) Thermal inkjet printing in tissue engineering and regenerative medicine. *Recent Patents on Drug Delivery & Formulation* 6(2):149–155.
55. Guillotin B et al. (2010) Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials* 31(28):7250–7256.
56. Ozbolat IT and Hospodiuk M (2016) Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials* 76:321–343.

57. Truby RL and Lewis JA (2016) Printing soft matter in three dimensions. *Nature* 540(7633):371–378.
58. Weinberger F, Mannhardt I, and Eschenhagen T (2017) Engineering cardiac muscle tissue. *Circulation Research* 120(9):1487–1500.
59. Jia J et al. (2014) Engineering alginate as bioink for bioprinting. *Acta Biomaterialia* 10(10):4323–4331.
60. Gao Q, He Y, Fu J-Z, Liu A, and Ma L (2015) Coaxial nozzle-assisted 3D bioprinting with built-in microchannels for nutrients delivery. *Biomaterials* 61:203–215.
61. Hockaday LA et al. (2012) Rapid 3D printing of anatomically accurate and mechanically heterogeneous aortic valve hydrogel scaffolds. *Biofabrication* 4(3):035005.
62. Zimmermann WH (2001) Tissue engineering of a differentiated cardiac muscle construct. *Circulation Research* 90(2):223–230.
63. Bian W, Badie N, Himel HD, and Bursac N (2014) Robust T-tubulation and maturation of cardiomyocytes using tissue-engineered epicardial mimetics. *Biomaterials* 35(12):3819–3828.
64. Pati F et al. (2014) Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. *Nature Communications* 5.
65. Kanzaki Y et al. (2010) Three-dimensional architecture of cardiomyocytes and connective tissue in human heart revealed by scanning electron microscopy. *Circulation* 122(19):1973–1974.
66. Jallerat Q, Szymanski JM, and Feinberg AW (2014) Nano- and microstructured ECM and biomimetic scaffolds for cardiac tissue engineering. *Bio-Inspired Materials for Biomedical Engineering*, (John Wiley & Sons, Inc.), pp. 195–226.
67. Drury JL and Mooney DJ (2003) Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials* 24(24):4337–4351.
68. Gaetani R et al. (2012) Cardiac tissue engineering using tissue printing technology and human cardiac progenitor cells. *Biomaterials* 33(6):1782–1790.
69. Gaetani R et al. (2015) Epicardial application of cardiac progenitor cells in a 3D-printed gelatin/hyaluronic acid patch preserves cardiac function after myocardial infarction. *Biomaterials* 61:339–348.
70. Zhang YS et al. (2016) Bioprinting 3D microfibrinous scaffolds for engineering endothelialized myocardium and heart-on-a-chip. *Biomaterials* 110:45–59.
71. Zhu K et al. (2017) Gold nanocomposite bioink for printing 3D cardiac constructs. *Advanced Functional Materials* 27(12):1605352.
72. Duan B, Hockaday LA, Kang KH, and Butcher JT (2013) 3D Bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *Journal of Biomedical Materials Research Part A* 101A(5):1255–1264.
73. Gaebel R et al. (2011) Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials* 32(35):9218–9230.
74. Homan KA et al. (2016) Bioprinting of 3D convoluted renal proximal tubules on perfusable chips. *Scientific Reports* 6(1).
75. Kolesky DB, Homan KA, Skylar-Scott MA, and Lewis JA (2016) Three-dimensional bioprinting of thick vascularized tissues. *Proceedings of the National Academy of Sciences of the United States of America* 113(12):3179–3184.
76. Compton BG and Lewis JA (2014) 3D-printing of lightweight cellular composites. *Advanced Materials* 26(34):5930–5935.
77. Martin JJ, Fiore BE, and Erb RM (2015) Designing bioinspired composite reinforcement architectures via 3D magnetic printing. *Nature Communications* 6:8641.
78. Sydney Gladman A, Matsumoto EA, Nuzzo RG, Mahadevan L, and Lewis JA (2016) Biomimetic 4D printing. *Nature Materials* 15(4):413–418.

79. Hinton TJ et al. (2015) Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances* 1(9).
80. Bhattacharjee T et al. (2015) Writing in the granular gel medium. *Science Advances* 1(8).
81. Hallett R, Moainie S, Hermiller J, and Fleischmann D (2016) CT and MRI of aortic valve disease: Clinical update. *Current Radiology Reports* 4(9):49.
82. Bonnichsen C and Ammash N (2016) Choosing between MRI and CT imaging in the adult with congenital heart disease. *Current Cardiology Reports* 18(5):45.
83. Tanabe Y et al. (2017) Three-dimensional maximum principal strain using cardiac computed tomography for identification of myocardial infarction. *European Radiology* 27(4):1667–1675.
84. Bramlet M, Olivieri L, Farooqi K, Ripley B, and Coakley M (2017) Impact of three-dimensional printing on the study and treatment of congenital heart disease. *Circulation Research* 120(6):904–907.
85. Nguyen K (2017) Past developments and future directions of 3D cardiac printing: A surgeon's perspective. *Rapid Prototyping in Cardiac Disease: 3D Printing the Heart*, Ed Farooqi KM (Springer International Publishing, Cham), pp. 183–189.
86. Vettukattil JJ, Samuel BP, Gosnell JM, and Kurup HKN (2017) Creation of a 3D printed model: From virtual to physical. *Rapid Prototyping in Cardiac Disease: 3D Printing the Heart*, Ed Farooqi KM (Springer International Publishing, Cham), pp. 9–19.
87. Batra S and Rakusan K (1992) Capillary length, tortuosity, and spacing in rat myocardium during cardiac cycle. *American Journal of Physiology-Heart and Circulatory Physiology* 263(5):H1369–H1376.
88. Cocosco CA et al. (2008) Automatic image-driven segmentation of the ventricles in cardiac cine MRI. *Journal of Magnetic Resonance Imaging* 28(2):366–374.
89. Bassar PJ and Pierpaoli C (2011) Microstructural and physiological features of tissues elucidated by quantitative-diffusion-tensor MRI. *Journal of Magnetic Resonance* 213(2):560–570.
90. Helm P, Beg MF, Miller MI, and Winslow RL (2005) Measuring and mapping cardiac fiber and laminar architecture using diffusion tensor MR imaging. *Annals of the New York Academy of Sciences* 1047(1):296–307.
91. Axel L (2017) Faster diffusion-weighted MR imaging of cardiac microstructure. *Radiology* 282(3):622–626.
92. Beard DA, Schenkman KA, and Feigl EO (2003) Myocardial oxygenation in isolated hearts predicted by an anatomically realistic microvascular transport model. *American Journal of Physiology-Heart and Circulatory Physiology* 285(5):H1826–H1836.
93. Hansen CJ et al. (2013) High-throughput printing via microvascular multinozzle arrays. *Advanced Materials* 25(1):96–102.
94. Kang H-W et al. (2016) A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology* 34(3):312–319.
95. Kolesky DB et al. (2014) 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials* 26(19):3124–3130.
96. Miller JS et al. (2012) Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials* 11(9):768–774.
97. Hinton TJ, Hudson A, Pusch K, Lee A, and Feinberg AW (2016) 3D printing PDMS elastomer in a hydrophilic support bath via freeform reversible embedding. *ACS Biomaterials Science & Engineering* 2:1781–1786.
98. Xin M, Olson EN, and Bassel-Duby R (2013) Mending broken hearts: Cardiac development as a basis for adult heart regeneration and repair. *Nature Review Molecular Cell Biology* 14(8):529–541.

99. Miller JS (2014) The billion cell construct: Will three-dimensional printing get us there? *PLoS Biology* 12(6):e1001882.
100. Sanchez PL et al. (2015) Acellular human heart matrix: A critical step toward whole heart grafts. *Biomaterials* 61:279–289.
101. Ott HC et al. (2008) Perfusion-decellularized matrix: Using nature’s platform to engineer a bioartificial heart. *Nature Medicine* 14(2):213–221.
102. Lu T-Y et al. (2013) Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. *Nature Communications* 4.
103. Yang X et al. (2014) Tri-iodo-L-thyronine promotes the maturation of human cardiomyocytes-derived from induced pluripotent stem cells. *Journal of Molecular and Cellular Cardiology* 72:296–304.
104. Radisic M, Marsano A, Maidhof R, Wang Y, and Vunjak-Novakovic G (2008) Cardiac tissue engineering using perfusion bioreactor systems. *Nature Protocols* 3(4):719–738.
105. Kocica MJ, Corno AF, Lackovic V, and Kanjuh VI (2007) The helical ventricular myocardial band of torrent-guasp. *Seminars in Thoracic and Cardiovascular Surgery: Pediatric Cardiac Surgery Annual* 10(1):52–60.
106. Kim DH et al. (2009) Nanoscale cues regulate the structure and function of macroscopic cardiac tissue constructs. *Proceedings of the National Academy of Sciences of the United States of America* 107(2):565–570.
107. Young AA, Legrice IJ, Young MA, and Smaill BH (1998) Extended confocal microscopy of myocardial laminae and collagen network. *Journal of Microscopy* 192(2):139–150.
108. Teh I et al. (2016) Resolving fine cardiac structures in rats with high-resolution diffusion tensor imaging. *Scientific Reports* 6(1):30573.
109. Srivastava D and Olson E (2000) A genetic blueprint for cardiac development. *Nature* 407: 221–226.
110. Ryckebusch L et al. (2008) Retinoic acid deficiency alters second heart field formation. *Proceedings of the National Academy of Sciences of the United States of America* 105(8):2913–2918.
111. Chu P-H, Ruiz-Lozano P, Zhoua Q, Chenleng C, and Chena J (2000) Expression patterns of FHL/SLIM family members suggest important functional roles in skeletal muscle and cardiovascular system. *Mechanisms of Development* 95:259–265.
112. Voronov DA, Alford PW, Xu G, and Taber LA (2004) The role of mechanical forces in dextral rotation during cardiac looping in the chick embryo. *Developmental Biology* 272(2):339–350.
113. Mohun TJ and Weninger WJ (2011) Imaging heart development using high-resolution episcopic microscopy. *Current Opinion in Genetics & Development* 21(5):573–578.
114. Taylor EN et al. (2016) Alterations in multi-scale cardiac architecture in association with phosphorylation of myosin binding protein-C. *Journal of the American Heart Association* 5(3):e002836.

Chapter 12 Additive manufacturing in the craniofacial area

Applications in vertical alveolar bone augmentation

Cedryck Vaquette, Kelly McGowan,
and Saso Ivanovski

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12.1 The alveolar bone, resorption, and current grafting techniques

12.1.1 Alveolar process

The alveolar process (or ridge) forms in the mandible and maxilla with the development and eruption of teeth. It is comprised of both supporting alveolar bone and alveolar bone proper (ABP), where ABP lines the socket and forms the attachment apparatus of the teeth in combination with the gingiva, periodontal ligament, and root cementum [1]. Loss of alveolar height and width occurs as a result of tooth loss, periodontal disease, infection, inflammation, trauma, and surgical resection of pathology [2]. The resulting dimensional changes [3–5] can present clinical challenges when attempting to restore the edentulous space with an implant-retained prosthesis.

12.1.2 Alveolar resorption

There is a wide variation in the degree of alveolar bone resorption experienced between patients, however some general patterns have been observed. Following the removal of a single tooth, Pietrokovski and Massler [6] found that hard and soft tissue resorption was twice as pronounced on the buccal aspect compared to palatal or lingual areas, and that specific teeth underwent more resorption than others. These findings were supported by a more recent study by Schropp et al. [7], which found that after 12 months, 50% of the alveolar ridge width was lost in addition to substantial bone height. Van der Weijden et al. [8] quantified the weighted mean bone changes after three months to be a 3.87 mm reduction in width and 1.67–2.03 mm in vertical bone loss, whereas at six months post extraction, Hammerle et al. [9] and Tan et al. [10] reported a 63% and 22% reduction in horizontal and vertical dimensions, respectively. The resulting bone loss is more severe where multiple teeth have been removed, with Bergman and Carlsson [11] demonstrating that most of the bone tissue of the alveolar process is eventually lost in these situations in the long term, as demonstrated by comparing profiles of the edentulous mandible and maxilla at 2 days, 5 years, and 21 years after tooth extraction. This means that patients who are missing multiple teeth and are the most likely to need dental reconstruction are also the ones who have the least amount of bone available to anchor implant-supported prosthesis.

12.1.3 Prevention of alveolar resorption

Consistent with the general treatment paradigm that prevention is better than a cure, a number of alveolar ridge preservation techniques have been developed to reduce post-extraction resorption [12,13], although no technique has yet been shown to prevent it entirely [14]. There is also limited evidence to support the

notion that interventions aimed at preserving the alveolar ridge at the time of extraction increase implant placement feasibility, or indeed that the survival, success, or marginal bone levels of implants placed following ridge preservation are superior compared to those undergoing unassisted healing [14].

12.1.4 Implant therapy in areas of alveolar resorption

Dental implant therapy is indicated to increase chewing comfort [15,16], to replace strategically important missing teeth [17], or to provide an alternative means of supporting a dental prosthesis where natural tooth and existing satisfactory restorations would otherwise be subjected to invasive preparation procedures [18,19]. Implants can be used to support a single tooth crown, a bridge, or fixed and removable full arch prosthesis, which makes them a valuable addition to the dental clinician's armamentarium. Safe and predictable implant placement requires an adequate volume of healthy bone, the availability of which must be carefully assessed through both clinical examination and three-dimensional (3D) radiography before commencing treatment [20]. The close proximity of the maxillary sinus and nasal cavity in the maxilla and the position of the inferior alveolar nerve within the mandible can be significant anatomical limitations to ideal implant placement and can act to limit the bone height available for implant placement following alveolar ridge resorption.

12.2 Augmenting resorbed alveolar bone

Alveolar bone augmentation procedures can be broadly divided into three categories:

- *Lateral augmentation*: [21] Via guided bone regeneration (GBR), particulate or block bone grafts, or the split osteotomy technique
- *Sinus floor elevation*: Via lateral elevation [22] or transalveolar access [23,24]
- *Vertical augmentation*: [25]

This current chapter is concerned with the restoration of the vertical dimension of the alveolar ridge and therefore will only address approaches focused on achieving vertical bone augmentation.

12.2.1 Vertical bone augmentation

Vertical bone augmentation can be achieved by a number of different techniques, as summarized in the following.

12.2.1.1 Guided bone regeneration GBR uses barrier membranes, with or without a supporting scaffold, for space maintenance and exclusion of the rapidly

invading soft tissues from the augmentation site in order to allow osteogenic cells to migrate and colonize the defect [26] as depicted in [Figure 12.1a](#). These barrier membranes can be either nonresorbable expanded polytetrafluoroethylene (e-PTFE) or resorbable, with both natural collagen and a variety of synthetic polymers available [27]. Although nonresorbable membranes function as more effective space maintainers and do not undergo any resorptive processes that could potentially interfere with bone formation, they require a second surgical procedure to remove and are associated with a higher incidence of soft tissue dehiscence and membrane exposure [28]. Exposure of the membrane is detrimental to the regenerative outcome, with 600% more new bone formation observed in sites where the membrane remained covered by overlying soft tissue [29]. Bioresorbable membranes are less stiff and tend to collapse, although this can be overcome with the use of a scaffold or graft material to maintain the desired space under the membrane, with similar bone regenerative outcomes demonstrated for nonresorbable membranes and collagen-resorbable membranes used with a scaffold [30]. Due to a lower rate of soft tissue dehiscence and the elimination of a second surgical procedure for removal, bioresorbable membranes are considered as the gold standard of treatment, provided they are used with a suitable graft material [20]. In general, GBR procedures display a high incidence of clinical success for intermediate-sized vertical bone deficiencies. In a systematic review of 74 patients with 220 implants, Rochietta et al. [25] found GBR to have a 0%–45% complication rate while experiencing gains of 2–8 mm of bone height with 1.27–2 mm subsequent loss over 1–7 year follow-up, and an overall implant survival rate of 92.1%–100% over 1–7 years.

12.2.1.2 Bone grafts and substitutes Autogenous bone as either a particulate or block graft is the current gold standard for bone regeneration [31,32]. Although their osteogenic properties are ideal, their resorption rate is high, their supply is limited, and harvesting requires a second surgery that is associated with post-operative pain and morbidities [33]. A number of bone substitutes have been proposed, including allografts harvested from cadaver donors, xenografts of animal origin, and synthetic alloplasts of calcium phosphates [34,35]. Synthetic particulate grafting materials present the advantages of no donor site morbidity and tailored degradation profiles allowing long-term space maintenance required for bone regeneration. Their significant drawback originates from their reduced osteogenic properties when compared to autologous bone, and their particulate nature means that handling and achieving stability can be challenging when large bone deficiencies need to be vertically augmented. These graft particles can also be combined with autologous bone to increase the bioactivity of these materials [32] as shown in [Figure 12.1b](#).

12.2.1.3 Distraction osteogenesis Distraction osteogenesis (DO) involves surgical separation of a bone segment from basal bone, with both segments then fixed to

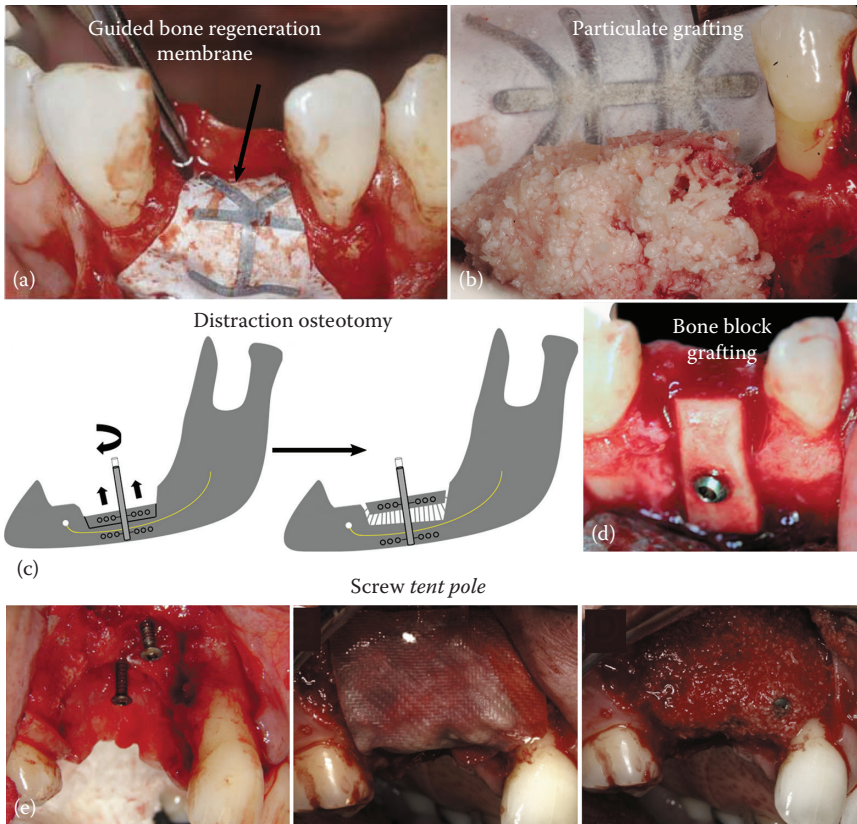


Figure 12.1 Current surgical techniques for vertical bone augmentation. (a) Guided bone regeneration using a titanium-reinforced nonresorbable ePTFE membrane. (From Hämmerle, C.H. and Jung, R.E., *Periodontology*, 2000, 33, 36–53, 2003. With permission), (b) utilization of a particulate bone graft for elevating the alveolar bone. (From Cordaro, L. et al., *Clin. Oral Implants Res.*, 13, 103–111, 2002. With permission), (c) a schematic describing the principles of distraction osteotomy. (From Rachmiel, A. et al., *J Oral Maxillofac. Surg.*, 75, 1164–1175, 2017. With permission), (d) autologous bone block graft whereby a piece of bone is fixed by means of a titanium screw onto the resident bone. (From Draenert, F. et al., *J. Biomed. Mater. Res. Part A*, 102, 1605–1613, 2014. With permission), and (e) screw tent pole technique allowing the creation of space for vertical bone augmentation. (From Le, B. et al., *J. Oral Maxillofac. Surg.*, 68, 428–435, 2010. With permission.)

an intraoral distractor [36,37] as depicted in [Figure 12.1c](#). In the afore-mentioned review by Rochietta et al. [25], DO was found to involve a 10%–75.7% complication rate, with some severe complications reported including loss of the entire separated segment. Although a 5–15 mm increase in vertical bone height was reported, additional grafting was required to correct undesirable palatal or lingual inclination in 41/91 patients. The survival rates of implants placed in sites augmented using DO were 90%–100%, with success rates ranging from 59% to 94%.

12.2.1.4 Onlay bone graft Onlay bone graft (OBG) is an autogenous bone grafting technique that requires harvesting of a block of bone from either an intraoral or extraoral donor site [38], which is thereafter grafted over the existing bone tissue, as shown in [Figure 12.1c](#). Intraoral sites usually include the mandibular ramus and mandibular symphysis, whereas extraoral grafts can be harvested from the iliac crest [39]. The graft can be positioned at the time of implant placement or 4–6 months before implant surgery. This procedure was reported by Rochietta et al. to undergo a 42% reduction in volume when not protected by a membrane, with implant survival 76%–100% [25]. Iliac crest grafts showed an average 4.22 mm bone height gain compared to 4.6 mm with intraoral blocks, with the iliac crest blocks also shown to undergo more extensive resorption. For these reasons, as well as comparative ease of access, intraoral sites are the preferred source of autografts.

As a variation to established clinical procedures, Le et al. [40] demonstrated a *tent-pole* grafting technique, where titanium screws were strategically placed in the alveolar bone leaving 5–7 mm of screw threads exposed above the ridge. Demineralized allograft was used to cover the screw completely, then a resorbable membrane was placed over the grafted sites as shown in [Figure 12.1e](#). Although it achieved sufficient bone augmentation to allow implant placement, it required release of the mylohyoid muscle from its attachment in the mandible and a split-thickness flap labially to avoid damage to the mental nerve. In all maxillary cases, the periosteal flaps were released from the nasal spine and scored to allow tension-free closure. The authors report that these additional methods are critical to avoid wound dehiscence and subsequent graft exposure.

Although able to provide an increase in vertical bone dimension, all of the most common bone grafting protocols are technically challenging and are complicated by the presence of anatomical limitations such as the sinus floor and inferior alveolar nerve [41], the requirement for multiple complex clinical procedures [42,43], and patient morbidity associated with secondary graft donor sites [31,44]. As a result, a significant body of research has been investigating the alternative methods to improve the vertical bone dimension and facilitate dental implant placement. The field of bone tissue engineering (BTE) is currently investigating many clinical

applications in the spinal, appendicular, and craniofacial regions of the skeleton [45,46]. Within the craniofacial complex, bioengineered bone has shown potential for periodontal regeneration, sinus augmentation, alveolar ridge preservation, and both lateral and vertical bones [33,47–49]. [Section 12.3](#) will elaborate the general requirements for successful BTE in the craniofacial region before narrowing that scope to additively manufactured approaches for vertical augmentation applications.

12.3 Principles of bone tissue engineering

Tissue engineering approaches to vertical bone augmentation have focused on the use of biomaterials to address the limitations of autogenous bone grafts. These biomaterials are defined as a “natural or synthetic material used to replicate part of a living system or function in intimate contact with living tissue” [33]. Compared to the BTE approaches developed for long bones, the craniofacial complex presents a unique set of clinical challenges. Masticatory function must be maintained, which makes immobilization for bone healing impractical and mandates that any successful augmentation procedure or material must be able to withstand the forces generated during chewing and swallowing. The important role of the head and neck region in social acceptance and self esteem must also be considered [50], as well as the impact of oral aesthetics and function on the patient’s quality of life [51]. The surgical environment may be further complicated by infection, irradiation, smoking, or systemic disease. The benefits of a tissue-engineered approach include reduced donor site morbidity, decreased technical difficulty of the surgical procedure, and shortened operative time [33]. The materials, cellular components, techniques, and considerations for BTE are outlined in the following.

12.3.1 Biocompatibility

Excellent biocompatibility is an essential criterion for a successful biomaterial [52]. The definition of biocompatibility incorporates a lack of immunological or clinically detectable primary or secondary foreign body reaction [53], the capacity to support normal cellular activity, molecular signalling without any local or systemic toxic effects to host tissue [54], and the ability to develop and sustain a mutually acceptable coexistence of biomaterials and host tissues [52]. Biomaterials are also assessed on their osteoinductive and osteoconductive properties, where the minimum requirement is osteoconduction but osteoinduction is preferred [55]. Osteoconduction is the property of a material that allows bone cells to adhere, proliferate, and form extracellular matrix (ECM) on its pores and surfaces [33]. Osteoinduction refers to the process by which osteogenesis is induced, specifically

the recruitment of immature cells and their subsequent differentiation into pre-osteoblasts. It is a crucial step in all bone-healing processes [56]. The ability of the scaffold to actively support nutrient, oxygen, and waste transport via the development of new blood vessels within weeks of implantation is also linked to its biocompatibility [54].

12.3.2 Bioresorbability

Scaffold materials for BTE should be bioresorbable, whereby bioresorption refers to the total elimination of the initial foreign material through natural pathways or simple filtration of degradation by-products [54,57]. This is an important distinction in which the use of the word bioresorption should be reserved for instances where total elimination has been shown conclusively [57]. In contrast, bioabsorbable materials can dissolve in body fluids without any polymer chain cleavage or molecular mass decrease; biodegradation refers to dispersion but with no proof of elimination from the body, whereas bioerodible refers to surface degradation only [57]. The rate of resorption is influenced by the material type, crystallinity, particle size, surface area, porosity, and the local biological environment [58]. Ideally, the rate of resorption would be adjusted by alteration of the above-mentioned principles to closely mimic the rate of new bone formation but not to exceed it. The ability to control the rate of resorption is highly desired.

12.3.3 Mechanical strength

An ideal scaffold must possess sufficient mechanical strength to maintain the structural integrity of the tissue, provide smooth load transfer to the native bone during normal function, and withstand the surgical implantation procedure [59]. The mechanical properties of bone differ significantly between cancellous and cortical types [60], which make defining the ideal mechanical properties of a BTE scaffold difficult. These differences are outlined in [Table 12.1](#).

The elastic properties of craniofacial bone are also influenced by species, age, anatomic location, and presence of the dentition [61]. The anisotropic elastic moduli for human mandibular cortical bone range from 11,000 to 30,000 MPa for the edentulous and dentate mandible, respectively [43]. Although it is suggested that scaffold mechanical properties should match those of the native

Table 12.1 Mechanical Properties of Cancellous and Cortical Bone

Mechanical Property	Cortical	Cancellous
Young's Modulus	15–20 GPa	0.1–2 GPa
Compressive Strength	100–200 MPa	2–20 MPa

Source: Olszta, M.J. et al., *Mater. Sci. Eng. R Rep.*, 58, 77–116, 2007.

tissue [62], there is little or no experimental data to suggest what the ideal values are for craniofacial BTE scaffolds.

12.3.4 Porosity

Sufficient scaffold porosity and interconnectivity are required for cell proliferation and differentiation, transport of nutrients, oxygen and waste, and the delivery of bioactive factors—all of which are required for effective tissue formation [59]. A pore size in the range of 200–350 μm has been reported as optimum for bone tissue ingrowth [63], and the combination of macro and microporosities has been shown to be more effective than macropores only [64]. However, increasing the porosity of the scaffold decreases its mechanical properties and complicates manufacturing and reproducibility [54]. The inversely proportionate relationship between mechanical strength and porosity is difficult to manage as high values of both porosity and mechanical strength would be ideal.

12.3.5 Biofactors

A successful scaffold should be able to effectively deliver proteins, cells, and genes—collectively termed *biofactors*—for the enhanced regeneration of natural tissue [59,65]. Although an ideal scaffold would provide all the necessary biofactors for bone regeneration in a spatially and temporally controlled manner, the extremely complex nature of their interactions with each other and the native tissue is neither fully understood nor able to be precisely replicated [66]. However, a number of proteins, stem cells, and platelets are known to participate in bone healing and their potential to augment bone regeneration has been examined. A number of growth factors have demonstrated potential for enhanced bone regeneration [67,68]. As a broad overview, a summary of potentially beneficial bone-specific signalling molecules is outlined in [Table 12.2](#).

Table 12.2 Growth Factors of Interest to Bone Engineering

Molecule	Abbreviation	Suggested Role
Transforming Growth Factor Beta	TGF- β	Triggers growth, differentiation, and ECM synthesis. TGF- β receptors are found in increased quantity in chondrocytes and osteoblasts. Functions as potential coupler between bone formation and resorption. Activates Runx2 expression.
Bone Morphogenetic Protein 2	BMP-2	Regulates osteoblast differentiation and stimulates bone formation. Acts as a dimer to type II & I transmembrane receptors, initiating a phosphorylation cascade upregulating bone-specific differentiation genes, controlling progression toward osteoblastic phenotype and matrix mineralization.
Bone Morphogenetic Protein 7	BMP-7	Osteoinductive—stimulates differentiation of mesenchymal stem cells to osteochondroblastic lineage. Suggested to exert action later (~2 weeks).
Fibroblast Growth Factor 1 (acidic)	FGF-1	Important for embryonic development, regeneration, wound healing, angiogenesis, and mesenchymal cell mitosis. Associated with chondrocyte proliferation. Increased activity detected in early stages of fracture healing.
Fibroblast Growth Factor 2 (basic)	FGF-2	Important for embryonic development, regeneration, wound healing, angiogenesis, and mesenchymal cell mitosis. Appears to be more potent than FGF-1. Is expressed by osteoblasts. Increased activity detected in early stages of fracture healing. Thought to play an important role in bone formation as regulator of Runx2 (osteogenesis-related transcription factor)
Insulin-like Growth Factor I	IGF I	Transduces signals via receptors, which have been located on both osteoblasts and osteoclasts. Has been suggested that IGF I supports formation and activation of osteoclasts.
Insulin-like Growth Factor II	IGF II	Transduces signals via receptors, which have been located on both osteoblasts and osteoclasts. Most abundant growth factor in bone, but less potent than IGF I. Has been localized in healing fractures.
Platelet-Derived Growth Factor BB	PDGF-BB	Secreted by platelets during early phase of fracture healing. Mitogenic for osteoblasts and has been localized at fracture sites. Transcriptionally increases osteoblasts VEGF mRNA expression at sites of skeletal injury to induce angiogenesis and promote fracture repair.
Vascular Endothelial Growth Factor	VEGF	Promotes angiogenesis, vasodilation, and increased microvascular permeability. Induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. Stimulates growth of collateral vessels with increased capillary density and blood flow.

Source: Hollinger, J.O. et al., *Bone Tissue Engineering*, CRC press, Boca Raton, FL, 2004.

12.4 Additive manufacturing for vertical bone augmentation

A variety of methods have been employed to fabricate porous bone scaffolds, including chemical/gas foaming, solvent casting, particle/salt leaching, freeze drying, thermally induced phase separation, and foam-gel [69]. However, none of these approaches are able to tailor pore size, shape, and interconnectivity—the importance of which was outlined in [Section 12.3](#). Additive manufacturing (AM) techniques have become increasingly popular as they allow precise control over the internal and external architecture and can be customised to each patient's clinical presentation [70].

All AM technologies involve the fabrication of a 3D object layer by layer [71] and include inkjet 3D printing, laser-assisted techniques, and extrusion 3D printing [70]. A key advantage of these techniques is their ability to design and fabricate scaffolds using the digital data from a patient's diagnostic imaging, either computer tomography (CT) or magnetic resonance imaging (MRI) [53,70]. An overview of each is provided in [Table 12.3](#).

AM has the potential to address many of the challenges faced when attempting to regenerate the complex anatomy of craniofacial structures [70]. Electrospinning is particularly promising, as it has been shown to fabricate nanofibrous scaffolds faster and more economically than traditional phase separation or self-assembly techniques and also allows a high degree of control over the structural and mechanical properties of the resulting scaffold [72]. Although each technique presents advantages and disadvantages, rigorous control over both scaffold exterior shape and interior porous architecture is essential for production of an effective scaffold for BTE [59]. This control is crucial to manage the balance between providing sufficient mechanical strength for load bearing and sufficient porosity for effective biofactor delivery and vascular ingrowth. A design technique that can also base the exterior shape of patient image data is desirable to produce custom-made scaffolds that fit the patient-specific defect [70].

Taking into consideration each of the above-mentioned criteria, it is evident that designing and manufacturing an ideal scaffold for BTE are complicated and multidisciplinary efforts.

The field of additive manufacturing is rapidly evolving and the first studies reporting on the utilization of additively manufactured scaffolds for vertical bone augmentation are very recent. Common to these studies is the utilization of bioceramic

Table 12.3 Overview of Additive Manufacturing Techniques for Bone Engineering Scaffolds

Method	Process	Limitations
Inkjet 3D Printing	Liquids or low-viscosity inks are deposited at predefined locations via a number of techniques (thermal, piezoelectric, and electromagnetic).	Shear stress of extrusion through print head can be limiting factor, and cell agglomeration or sedimentation can reduce print quality.
Laser-Assisted	Generally consist of pulsed laser source, a transparent glass slide covered with a layer of cells and biomaterials, and a receiving substrate in a number of systems (biological laser printing, selective laser sintering, stereolithography, absorbing film-assisted).	Techniques involving heat are not compatible with living cells. Some issues have been reported with removal of loose powder post-processing and extensive cracking in scaffolds with nonuniform porosity.
Extrusion 3D Printing	Fused deposition modeling (FDM) involves melting a premade filament and extruding the molten-state polymer onto a collector or substrate. Other types of extrusion printers use a mechanical action (piston) or pneumatic system (compressed air). Direct melt electrospinning writing (MEW) forces the extruded polymer solution or melt through an electrical field (<10 kV), capable of producing micro- or nanofibers.	Requirement for molten state in FDM restricts suitable materials. However, recent developments in electrospinning appear promising: This technique is able to produce 3D scaffolds with systematic variations in morphology, often incorporating both nano- and microfibres for improved outcomes in bone tissue engineering.

Source: Bose, S. et al., *Mater. Today*, 16, 496–504, 2013; Obregon, F. et al., *J. Dent. Res.*, 94, 143S–152S, 2015.

for the purpose of maintaining space and concomitantly delivering calcium and phosphate entities to trigger or guide bone formation.

One of the first reports combined 3D powder-printing techniques and calcium phosphate cement chemistry [73]. This was achieved by reacting a mixture of α/β tri-calcium phosphate with phosphoric acid to obtain a solid bioceramic scaffold that is mostly composed of a calcium phosphate phase called monetite. It was demonstrated that good shape and dimensional printing accuracy were obtained as shown in [Figure 12.2a](#). This technique circumvented many of the drawbacks associated with the manufacturing of complex, patient-specific bioceramic scaffolds using conventional techniques such as sintering (heat treatment). Indeed, the direct reaction of

the acid with the ceramic powder enabled the solidification of the powder creating a self-standing scaffold with compressive strengths in the MPa range while avoiding dimensional changes observed in ceramics during sintering for achieving this same purpose. This also improved the degradability of the bioceramic scaffold, which had fully degraded 56 weeks post-intramuscular implantation in rats. However, the resulting scaffold had a relatively low porosity ranging from 30% to 50% and a randomly organized porous network (Figure 12.2b) with small pore size that can be detrimental for tissue colonization, neovascularization, and ultimately for bone formation.

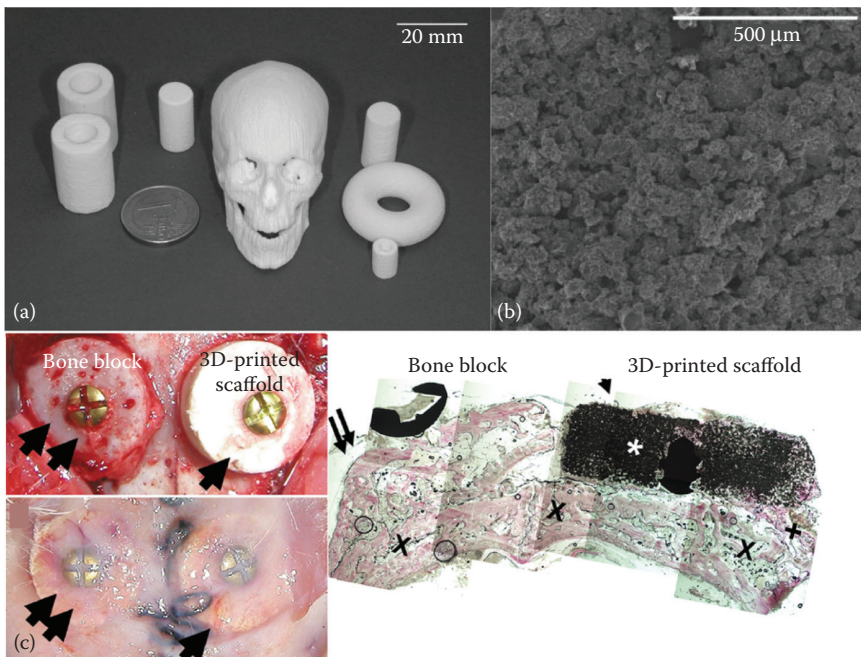


Figure 12.2 Additive manufacturing for vertical bone augmentation using powder bed 3D printer. (a) Customized shapes made of calcium phosphate materials (Monetite) manufactured by contacting the powder material with a solvent to initiate fusion of the particle and hence enabling printing. (b) Internal scaffold architecture featuring low porosity, small pore sizes, and randomly organized porous network. (From Gbureck, U. et al., *Adv. Funct. Mater.*, 17, 3940–3945, 2007. With permission.) (c) *In vivo* performance of the 3D-printed scaffold in comparison to autologous bone in an extraskeletal lapine vertical bone augmentation model, showing the scaffold fixation upon implantation and the regenerative outcome 8 weeks post-surgery, heterogeneous new bone formation mostly located on the periphery of the scaffold was observed. (From Tamimi, F. et al., *Biomaterials*, 30, 6318–6326, 2009. With permission.) (Continued)

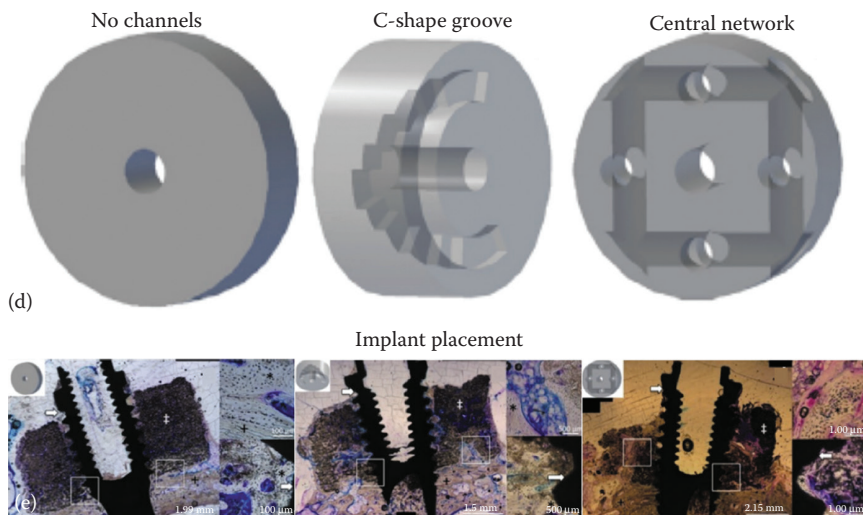


Figure 12.2 (Continued) Additive manufacturing for vertical bone augmentation using powder bed 3D printer. (d) Subsequent designs including perfusion channels for enhancing vascularization and bone formation, and (e) this enabled increased osteogenesis that permitted implant placement; primary stability was achieved even though bone-to-implant contact remained moderate. (From Tamimi, F. et al., *Biomaterials*, 35, 5436–5445, 2014. With permission.)

Subsequent studies investigated the performance of this 3D-printed synthetic bone-grafting scaffold in a rabbit calvarial extraskeletal vertical bone formation model and demonstrated that bone height of up to 4 mm could be gained 8 weeks post implantation [74]. Despite a relatively significant bone fill within the scaffold of around 40%, the bone distribution remained highly heterogeneous and was mostly located in the direct vicinity of the resident calvarial bone and on the periphery of the scaffold (where maximal bone height was observed). This gradient in the bone formation pattern was strongly linked with the level of vascularization, which was not sufficient in the most central portion of the 3D-printed scaffold.

The degradation of the monetite scaffold was investigated in a similar extraskeletal animal model and compared to both the osteogenic performance and the resorption pattern of a conventional autologous onlay bone block graft, as shown on [Figure 12.2c](#). It was demonstrated that half of the synthetic scaffold has resorbed within the 8 weeks of the implantation period, whereas the autologous bone graft displayed a significantly increased resorption rate. However, the resorption or material degradation was more pronounced on the lateral aspects of the onlay grafts, as shown in [Figure 12.2c](#). The periphery of the onlay graft was the location of

preferential bone formation in contrast to the inner sections of the scaffold/ autologous block, which did not feature a great deal of osteogenesis [75]. A subsequent refinement of the scaffold's design included the incorporation of channels created during the printing process, aimed at enhancing the level of blood perfusion and neovascularization and therefore improving subsequent bone formation [76]. Several shapes and arrangements were tested, including the inclusion of a C-shaped channel and a central network composed of eight interconnected channels (four vertical and four horizontal) opening into all of the surfaces of the 3D scaffold, as shown in [Figure 12.2d](#). The latter design resulted in a significantly improved regenerative outcome as more homogenous and increased bone volume was observed for this group. The presence of these channels also impacted the resorption pattern of the graft as 60% of the monetite had degraded over the 12 weeks of the implantation time period (as opposed to 40% without channels). A further step toward clinical relevance was also taken by performing a surgical reentry for the purpose of implant placement ([Figure 12.2e](#)). Although the presence of the channels had a strong impact on the bone formation pattern and distribution, this effect was not fully translated to a benefit for dental implant osteointegration. Indeed, no strong differences were noticed between the different designs and it was hypothesized that the channels were physically too distant to affect bone to implant contact. These proofs of concept studies were the first steps toward the development of customized patient-specific 3D-printed scaffold for vertical bone augmentation. They demonstrated several key points: (1) Osteogenesis could occur within the synthetic graft and a homogenous bone distribution could be achieved via the incorporation of channels in the inner section of the scaffold; (2) the 3D-printed scaffolds could withstand the surgical handling including the placement of a fixation screw to biomechanically stabilize the construct (which is of high significance for clinical application as bioceramic scaffolds are usually brittle); and (3) the scaffold permitted implant placement, which is the ultimate goal of vertical bone augmentation. Despite these significant and positive outcomes, these scaffolds have the drawbacks of a low porosity and small pore size, which drastically prevents tissue infiltration, vascularization, and hence complete bone regeneration.

These drawbacks were circumvented by utilizing a different printing technology that enabled a better control over the pore size and porous network arrangement. In a series of three recent papers [77–79], a direct filament printing technology was utilized based on the extrusion of a highly viscous tricalcium phosphate α -TCP, microcrystalline, and calcium-deficient HA paste, which sets to a hard consistency. This resulted in the manufacturing of a highly porous scaffold with a precisely controlled pore size and architecture, as shown in [Figure 12.3a](#). The performance toward vertical bone augmentation was tested using an extraskeletal bone formation model in sheep using the following protocol: A peripheral groove was created to facilitate the placement of an occlusive titanium dome into which the 3D-printed scaffold was placed. Several perforations were also performed into the calvarial bone

to enable the formation of a blood clot under the elevated space below the dome (Figure 12.3b). In this particular experimental setting, substantial bone formation 8 weeks post implantation was observed in the scaffold, as shown in Figure 12.3c, which outperformed, in the early phase of healing, the performance of two different commercially available particulate bone-grafting products (Ceros made of β -TCP, and Bio-oss composed of particulate bovine bone) [78]. These excellent osteoconductive properties originated from the highly organized architecture of the scaffold featuring an interconnected porosity with macroscopic pore size of 250 μm , which was sufficiently high to enable appropriate vascularization and therefore subsequent bone formation. This concept was further investigated using an osteogenic cue, Bone Morphogenetic Protein-2 (BMP-2), impregnated into the porous 3D-printed scaffold [79]. This presents the obvious advantage of not only relying on the healing capacity of the patient, which in elderly patients can lead to poor regeneration. The incorporation of BMP-2 within the porous ceramic scaffold induced substantial bone formation as early as 8 weeks post implantation, and the amount of newly formed bone was, as expected, greater in the BMP-2-loaded scaffold than in the corresponding negative control without the osteogenic protein. This trend was confirmed at the 16-week time point, which showed that the BMP-2-loaded group achieved bone

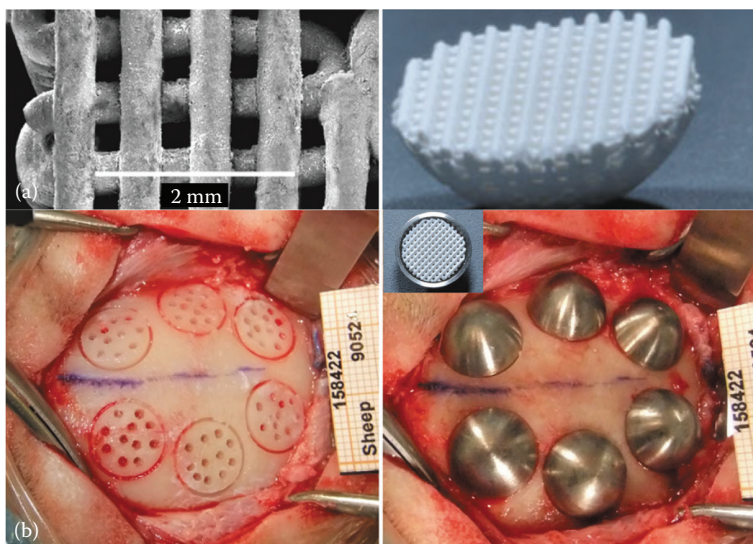


Figure 12.3 3D-printed scaffold based on extrusion printing. (a) Morphology of the scaffold manufactured by extruding a viscous calcium phosphate cement. The resulting construct featured an interconnected porous network essential for bone formation. (b) Surgical protocol for assessing the scaffold performance using an extraskeletal calvarial bone formation model in sheep. The scaffold was placed into a titanium cup to prevent soft tissue infiltration. *(Continued)*

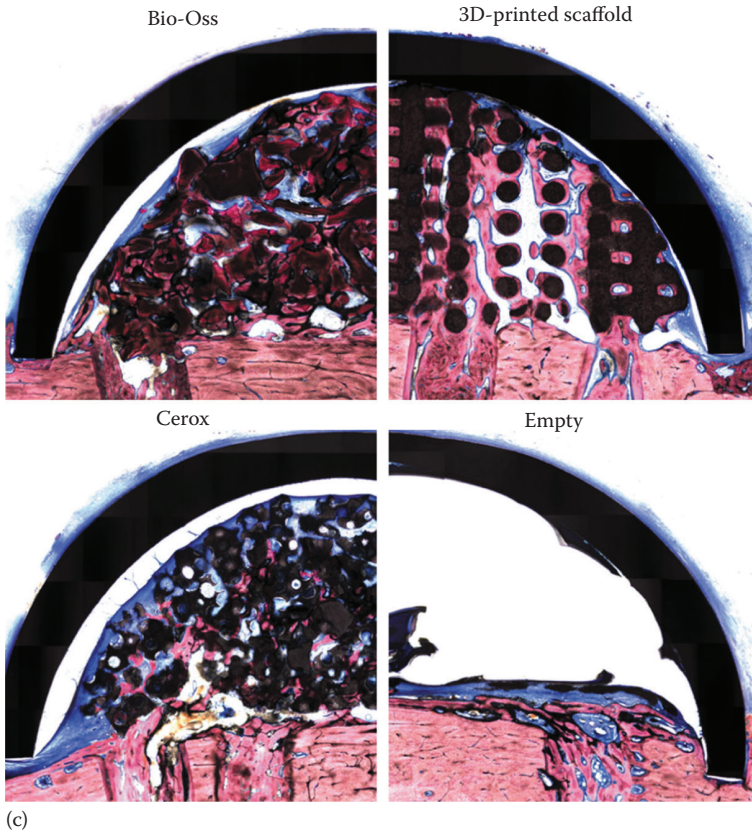


Figure 12.3 (Continued) 3D-printed scaffold based on extrusion printing.
(c) Regenerative outcomes 8 weeks post-implantation demonstrating significantly increased bone formation when compared to commercially available particulate materials. (From Carrel, J.-P. et al., *Clin Oral Implants Res.*, 27, 55–62, 2016. With permission.)

levels approaching full bone fill, characterized by the presence of newly formed osteons, harversian-like structures, and adipocytic bone marrow. More surprisingly, a differential resorption pattern was observed in the presence of the BMP-2, as the synthetic bioceramic experienced a 2.7-fold increase in the resorption rate resulting in nearly full degradation of the 3D-printed scaffold.

Although these studies demonstrated the potential of the bioceramic 3D-printed scaffold for vertical bone formation, the implantation site was not clinically relevant. In a subsequent pilot study, involving the placement of the scaffold in a surgically created edentulous space in one beagle dog ([Figure 12.4a](#) and [b](#)), Carrel et al. [77] demonstrated that significant vertical bone formation in the mandible could be achieved

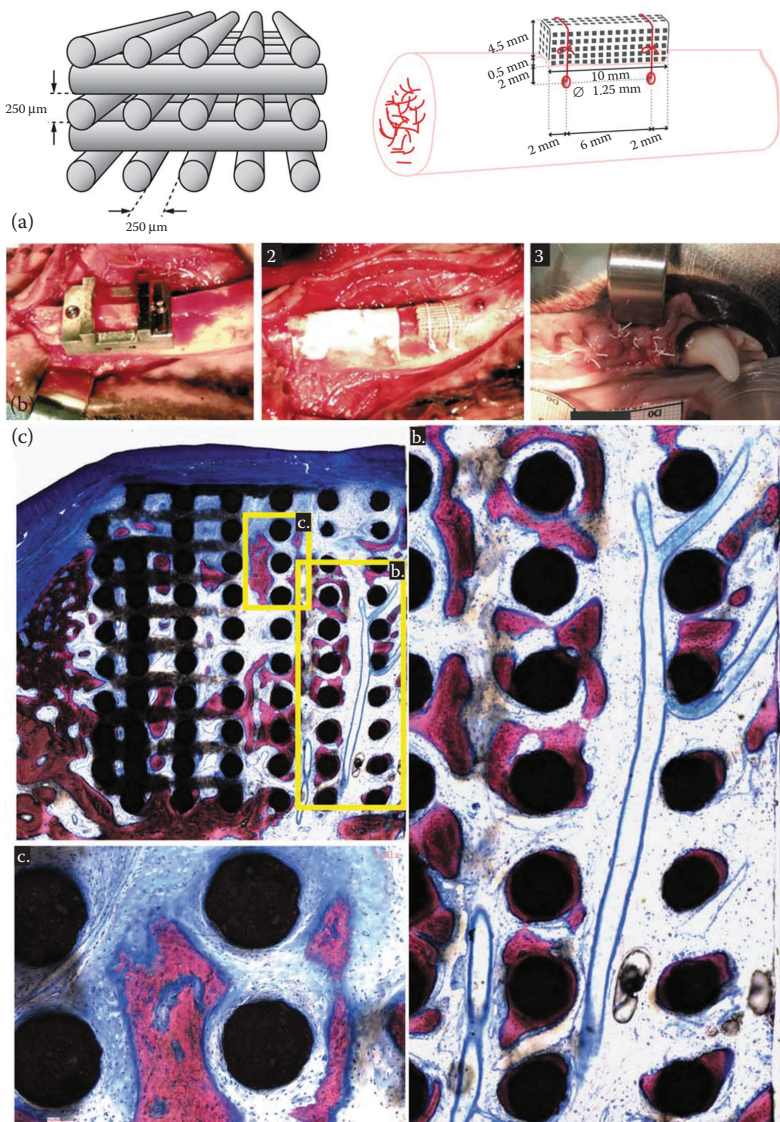


Figure 12.4 Vertical bone augmentation in a dog mandible, a clinically relevant site. (a) A schematic depicting the scaffold architecture and the implantation site, (b) surgical approach featuring bone bed preparation and scaffold placement, which was covered with a collagen membrane according to the principles of guided bone regeneration and suturing the flap, and (c) bone elevation outcome demonstrating a 4.5 mm gain in height in several locations of the scaffold. (From Carrel, J.-P. et al., *Clin Implant Dent Relat Res.*, 18, 1183–1192, 2016. With permission.)

using the bioceramic scaffold (Figure 12.4c). Indeed, these authors observed vertical bone augmentation up to 4.5 mm height in their 5 mm thick scaffold.

12.5 Conclusion

This chapter has described the current techniques for vertical bone augmentation and has highlighted the need for the development of bioengineering products to circumvent the shortcomings of these strategies. These products must fulfill a certain number of criteria that have been detailed, and AM can strongly contribute to the development of construct with significant bench to bedside translation. Hence vertical bone formation seems to be predictably achieved using additively manufactured scaffolds, however significant issues are not yet resolved including the issue of soft tissue closure after scaffold placement. If not controlled, this can ultimately result in soft tissue dehiscence leading to graft exposure and failure of the regenerative procedure. Another aspect, rarely addressed in the literature, is the long-term fate of the bone subsequent to surgical reentry and dental implant placement. Resorption of the regenerated bone can be a challenge, as already seen in the current clinical setting, especially when autogenous grafting is used.

References

1. M. Listgarten, Normal development, structure, physiology and repair of gingival epithelium, *Oral Sciences Reviews* 1 (1971) 3–67.
2. M.S. Tonetti, C.H. Hämmeler, Advances in bone augmentation to enable dental implant placement: Consensus Report of the Sixth European Workshop on Periodontology, *Journal of Clinical Periodontology* 35(s8) (2008) 168–172.
3. D.A. Atwood, Reduction of residual ridges: A major oral disease entity, *The Journal of Prosthetic Dentistry* 26(3) (1971) 266–279.
4. G. Cardaropoli, M. Araujo, J. Lindhe, Dynamics of bone tissue formation in tooth extraction sites, *Journal of Clinical Periodontology* 30(9) (2003) 809–818.
5. M. Farmer, I. Darby, Ridge dimensional changes following single-tooth extraction in the aesthetic zone, *Clinical Oral Implants Research* 25(2) (2014) 272–277.
6. J. Piotrowski, M. Massler, Alveolar ridge resorption following tooth extraction, *The Journal of Prosthetic Dentistry* 17(1) (1967) 21–27.
7. L. Schropp, A. Wenzel, L. Kostopoulos, T. Karring, Bone healing and soft tissue contour changes following single-tooth extraction: A clinical and radiographic 12-month prospective study, *International Journal of Periodontics and Restorative Dentistry* 23(4) (2003) 313–324.
8. F. Van der Weijden, F. Dell’Acqua, D.E. Slot, Alveolar bone dimensional changes of post-extraction sockets in humans: A systematic review, *Journal of Clinical Periodontology* 36(12) (2009) 1048–1058.
9. C.H. Hämmeler, M.G. Araújo, M. Simion, Evidence-based knowledge on the biology and treatment of extraction sockets, *Clinical Oral Implants Research* 23(s5) (2012) 80–82.

10. W.L. Tan, T.L. Wong, M. Wong, N.P. Lang, A systematic review of post-extractional alveolar hard and soft tissue dimensional changes in humans, *Clinical Oral Implants Research* 23(s5) (2012) 1–21.
11. B. Bergman, G.E. Carlsson, Clinical long-term study of complete denture wearers, *The Journal of Prosthetic Dentistry* 53(1) (1985) 56–61.
12. F. Vignoletti, P. Matesanz, D. Rodrigo, E. Figuero, C. Martin, M. Sanz, Surgical protocols for ridge preservation after tooth extraction. A systematic review, *Clinical Oral Implants Research* 23(s5) (2012) 22–38.
13. R.E. Wang, N.P. Lang, Ridge preservation after tooth extraction, *Clinical Oral Implants Research* 23(s6) (2012) 147–156.
14. N. Mardas, A. Trullenque-Eriksson, N. MacBeth, A. Petrie, N. Donos, Does ridge preservation following tooth extraction improve implant treatment outcomes: A systematic review, *Clinical Oral Implants Research* 26(S11) (2015) 180–201.
15. F. Fontijn-Tekamp, A. Slagter, A. Van Der Bilt, M.V.T. Hof, D. Witter, W. Kalk, J. Jansen, Biting and chewing in overdentures, full dentures, and natural dentitions, *Journal of Dental Research* 79(7) (2000) 1519–1524.
16. F.A. Fontijn-Tekamp, A.P. Slagter, A. Van der Bilt, M.A. Van't Hof, W. Kalk, J.A. Jansen, Swallowing thresholds of mandibular implant-retained overdentures with variable portion sizes, *Clinical Oral Implants Research* 15(3) (2004) 375–380.
17. N.U. Zitzmann, G. Krastl, H. Hecker, C. Walter, T. Waltimo, R. Weiger, Strategic considerations in treatment planning: Deciding when to treat, extract, or replace a questionable tooth, *The Journal of Prosthetic Dentistry* 104(2) (2010) 80–91.
18. B.E. Pjetursson, U. Brägger, N.P. Lang, M. Zwahlen, Comparison of survival and complication rates of tooth-supported fixed dental prostheses (FDPs) and implant-supported FDPs and single crowns (SCs), *Clinical Oral Implants Research* 18(s3) (2007) 97–113.
19. K. Tan, B.E. Pjetursson, N.P. Lang, E.S. Chan, A systematic review of the survival and complication rates of fixed partial dentures (FPDs) after an observation period of at least 5 years, *Clinical Oral Implants Research* 15(6) (2004) 654–666.
20. N.P. Lang, J. Lindhe, *Clinical Periodontology and Implant Dentistry*, 2 Volume Set, John Wiley & Sons, Chichester, England, 2015.
21. N. Donos, N. Mardas, V. Chadha, Clinical outcomes of implants following lateral bone augmentation: Systematic assessment of available options (barrier membranes, bone grafts, split osteotomy), *Journal of Clinical Periodontology* 35(s8) (2008) 173–202.
22. B.E. Pjetursson, W.C. Tan, M. Zwahlen, N.P. Lang, A systematic review of the success of sinus floor elevation and survival of implants inserted in combination with sinus floor elevation, *Journal of Clinical Periodontology* 35(s8) (2008) 216–240.
23. R.B. Summers, A new concept in maxillary implant surgery: The osteotome technique, *Compendium* (Newtown, Pa.) 15(2) (1994) 152, 154–156.
24. W.C. Tan, N.P. Lang, M. Zwahlen, B.E. Pjetursson, A systematic review of the success of sinus floor elevation and survival of implants inserted in combination with sinus floor elevation Part II: Transalveolar technique, *Journal of Clinical Periodontology* 35(s8) (2008) 241–254.
25. I. Rocchietta, F. Fontana, M. Simion, Clinical outcomes of vertical bone augmentation to enable dental implant placement: A systematic review, *Journal of Clinical Periodontology* 35(s8) (2008) 203–215.
26. C. Dahlin, A. Linde, J. Gottlow, S. Nyman, Healing of bone defects by guided tissue regeneration, *Plastic and Reconstructive Surgery* 81(5) (1988) 672–676.
27. R.E. Jung, R. Zwahlen, F.E. Weber, A. Molenberg, G.H. Van Lenthe, C.H. Hammerle, Evaluation of an in situ formed synthetic hydrogel as a biodegradable membrane for guided bone regeneration, *Clinical Oral Implants Research* 17(4) (2006) 426–433.

28. C.H. Hämmerle, R.E. Jung, Bone augmentation by means of barrier membranes, *Periodontology* 2000 33(1) (2003) 36–53.
29. E.E. Machtei, The effect of membrane exposure on the outcome of regenerative procedures in humans: A meta-analysis, *Journal of Periodontology* 72(4) (2001) 512–516.
30. M.B. Hürzeler, R.J. Kohal, J. Naghshbandi, L.F. Mota, J. Conradt, D. Hutmacher, R.G. Caffesse, Evaluation of a new bioresorbable barrier to facilitate guided bone regeneration around exposed implant threads: An experimental study in the monkey, *International Journal of Oral and Maxillofacial Surgery* 27(4) (1998) 315–320.
31. E. Neovius, T. Engstrand, Craniofacial reconstruction with bone and biomaterials: Review over the last 11 years, *Journal of Plastic, Reconstructive & Aesthetic Surgery* 63(10) (2010) 1615–1623.
32. L. Cordaro, D.S. Amadè, M. Cordaro, Clinical results of alveolar ridge augmentation with mandibular block bone grafts in partially edentulous patients prior to implant placement, *Clinical Oral Implants Research* 13(1) (2002) 103–111.
33. R. Tevlin, A. McArdle, D. Atashroo, G. Walmsley, K. Senarath-Yapa, E. Zielins, K. Paik, M. Longaker, D. Wan, Biomaterials for craniofacial bone engineering, *Journal of Dental Research* 93(12) (2014) 1187–1195.
34. N. Baldini, M. De Sanctis, M. Ferrari, Deproteinized bovine bone in periodontal and implant surgery, *Dental Materials* 27(1) (2011) 61–70.
35. R.A. Yukna, Synthetic bone grafts in periodontics, *Periodontology* 2000 1(1) (1993) 92–99.
36. M. Chiapasco, U. Consolo, A. Bianchi, P. Ronchi, Alveolar distraction osteogenesis for the correction of vertically deficient edentulous ridges: A multicenter prospective study on humans, *International Journal of Oral & Maxillofacial Implants* 19(3) (2004) 399–407.
37. A. Rachmiel, D. Shilo, D. Aizenbud, O. Emodi, Vertical alveolar distraction osteogenesis of the atrophic posterior mandible before dental implant insertion, *Journal of Oral and Maxillofacial Surgery* 75 (2017) 1164–1175.
38. C. Myeroff, M. Archdeacon, Autogenous bone graft: Donor sites and techniques, *Journal of Bone and Joint Surgery American* 93(23) (2011) 2227–2236.
39. F. Draenert, D. Huetzen, A. Neff, W. Mueller, Vertical bone augmentation procedures: Basics and techniques in dental implantology, *Journal of Biomedical Materials Research Part A* 102(5) (2014) 1605–1613.
40. B. Le, M.D. Rohrer, H.S. Prasad, Screw “tent-pole” grafting technique for reconstruction of large vertical alveolar ridge defects using human mineralized allograft for implant site preparation, *Journal of Oral and Maxillofacial Surgery* 68(2) (2010) 428–435.
41. M. Esposito, M.G. Grusovin, P. Felice, G. Karatzopoulos, H.V. Worthington, P. Coulthard, The efficacy of horizontal and vertical bone augmentation procedures for dental implants—a Cochrane systematic review, *European Journal of Oral Implantology* 2(3) (2009) 167–184.
42. M. Chiapasco, M. Zaniboni, L. Rimondini, Autogenous onlay bone grafts vs. alveolar distraction osteogenesis for the correction of vertically deficient edentulous ridges: A 2–4-year prospective study on humans, *Clinical Oral Implants Research* 18(4) (2007) 432–440.
43. I. Urban, A. Monje, H. Wang, Vertical ridge augmentation and soft tissue reconstruction of the anterior atrophic maxillae: A case series, *The International Journal of Periodontics and Restorative Dentistry* 35 (2015) 613–623.
44. T. Iizuka, W. Smolka, W. Hallermann, R. Mericske-Stern, Extensive augmentation of the alveolar ridge using autogenous calvarial split bone grafts for dental rehabilitation, *Clinical Oral Implants Research* 15(5) (2004) 607–615.
45. A. Oryan, S. Alidadi, A. Moshiri, N. Maffulli, Bone regenerative medicine: Classic options, novel strategies, and future directions, *Journal of Orthopaedic Surgery and Research* 9(1) (2014) 1.

46. M.M. Stevens, Biomaterials for bone tissue engineering, *Materials Today* 11(5) (2008) 18–25.
47. E. Scheller, P. Krebsbach, D. Kohn, Tissue engineering: State of the art in oral rehabilitation, *Journal of Oral Rehabilitation* 36(5) (2009) 368–389.
48. B. Ward, S. Brown, P. Krebsbach, Bioengineering strategies for regeneration of craniofacial bone: A review of emerging technologies, *Oral Diseases* 16(8) (2010) 709–716.
49. Y. Xiao, Bone tissue engineering for dentistry and orthopaedics, *BioMed Research International* 2014 (2014) 241067.
50. G.R. Adams, The effects of physical attractiveness on the socialization process, Psychological aspects of facial form. *Craniofacial Growth Series Monograph* (11) (1981) 25–47.
51. E.C.D. Wearers, Correlation between quality of life and denture satisfaction in elderly complete denture wearers, *The International Journal of Prosthodontics* 14(1) (2001) 77.
52. D.F. Williams, On the mechanisms of biocompatibility, *Biomaterials* 29(20) (2008) 2941–2953.
53. D.W. Hutmacher, Scaffold design and fabrication technologies for engineering tissues—state of the art and future perspectives, *Journal of Biomaterials Science; Polymer Edition* 12(1) (2001) 107–124.
54. S. Bose, M. Roy, A. Bandyopadhyay, Recent advances in bone tissue engineering scaffolds, *Trends in Biotechnology* 30(10) (2012) 546–554.
55. V. Petrovic, P. Zivkovic, D. Petrovic, V. Stefanovic, Craniofacial bone tissue engineering, *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* 114(3) (2012) e1–e9.
56. T. Albrektsson, C. Johansson, Osteoinduction, osteoconduction and osseointegration, *European Spine Journal* 10(2) (2001) S96–S101.
57. M. Vert, S. Li, G. Spenlehauer, P. Guérin, Bioresorbability and biocompatibility of aliphatic polyesters, *Journal of Materials Science: Materials in Medicine* 3(6) (1992) 432–446.
58. D. Hutmacher, M.B. Hürzeler, H. Schliephake, A review of material properties of biodegradable and bioresorbable polymers and devices for GTR and GBR applications, *International Journal of Oral & Maxillofacial Implants* 11(5) (1996) 667–678.
59. S.J. Hollister, C. Lin, E. Saito, R. Schek, J. Taboas, J. Williams, B. Partee, C. Flanagan, A. Diggs, E. Wilke, Engineering craniofacial scaffolds, *Orthodontics & Craniofacial Research* 8(3) (2005) 162–173.
60. M.J. Olszta, X. Cheng, S.S. Jee, R. Kumar, Y.-Y. Kim, M.J. Kaufman, E.P. Douglas, L.B. Gower, Bone structure and formation: A new perspective, *Materials Science and Engineering: R: Reports* 58(3) (2007) 77–116.
61. C. Schwartz-Dabney, P. Dechow, Edentulation alters material properties of cortical bone in the human mandible, *Journal of Dental Research* 81(9) (2002) 613–617.
62. D.W. Hutmacher, Scaffolds in tissue engineering bone and cartilage, *Biomaterials* 21(24) (2000) 2529–2543.
63. C.M. Murphy, M.G. Haugh, F.J. O'Brien, The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering, *Biomaterials* 31(3) (2010) 461–466.
64. J.R. Woodard, A.J. Hildore, S.K. Lan, C. Park, A.W. Morgan, J.A.C. Eurell, S.G. Clark, M.B. Wheeler, R.D. Jamison, A.J.W. Johnson, The mechanical properties and osteoconductivity of hydroxyapatite bone scaffolds with multi-scale porosity, *Biomaterials* 28(1) (2007) 45–54.
65. J. Hollinger, Factors for osseous repair and delivery: Part II, *Journal of Craniofacial Surgery* 4(3) (1993) 135–141.

66. J.O. Hollinger, T.A. Einhorn, B. Doll, C. Sfeir, *Bone Tissue Engineering*, CRC press, Boca Raton, FL, 2004.
67. J.E. Cillo, R. Gassner, R.R. Koepsel, M.J. Buckley, Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: Implications for distraction osteogenesis, *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 90(2) (2000) 147–154.
68. R.E. Jung, D.S. Thoma, C.H. Hammerle, Assessment of the potential of growth factors for localized alveolar ridge augmentation: A systematic review, *Journal of Clinical Periodontology* 35(s8) (2008) 255–281.
69. S. Bose, S. Vahabzadeh, A. Bandyopadhyay, Bone tissue engineering using 3D printing, *Materials Today* 16(12) (2013) 496–504.
70. F. Obregon, C. Vaquette, S. Ivanovski, D. Hutmacher, L. Bertassoni, Three-dimensional bioprinting for regenerative dentistry and craniofacial tissue engineering, *Journal of Dental Research* 94 (2015) 143S–152S.
71. B. Derby, Printing and prototyping of tissues and scaffolds, *Science* 338(6109) (2012) 921–926.
72. W.J. Li, C.T. Laurencin, E.J. Caterson, R.S. Tuan, F.K. Ko, Electrospun nanofibrous structure: A novel scaffold for tissue engineering, *Journal of Biomedical Materials Research* 60(4) (2002) 613–621.
73. U. Gbureck, T. Hölzel, U. Klammert, K. Würzler, F.A. Müller, J.E. Barralet, Resorbable dicalcium phosphate bone substitutes prepared by 3D powder printing, *Advanced Functional Materials* 17(18) (2007) 3940–3945.
74. J. Torres, F. Tamimi, M.H. Alkhraisat, J.C. Prados-Frutos, E. Rastikerdar, U. Gbureck, J.E. Barralet, E. López-Cabarcos, Vertical bone augmentation with 3D-synthetic monetite blocks in the rabbit calvaria, *Journal of Clinical Periodontology* 38(12) (2011) 1147–1153.
75. F. Tamimi, J. Torres, U. Gbureck, E. Lopez-Cabarcos, D.C. Bassett, M.H. Alkhraisat, J.E. Barralet, Craniofacial vertical bone augmentation: A comparison between 3D printed monolithic monetite blocks and autologous onlay grafts in the rabbit, *Biomaterials* 30(31) (2009) 6318–6326.
76. F. Tamimi, J. Torres, K. Al-Abedalla, E. Lopez-Cabarcos, M.H. Alkhraisat, D.C. Bassett, U. Gbureck, J.E. Barralet, Osseointegration of dental implants in 3D-printed synthetic onlay grafts customized according to bone metabolic activity in recipient site, *Biomaterials* 35(21) (2014) 5436–5445.
77. J.-P. Carrel, A. Wiskott, S. Scherrer, S. Durual, Large bone vertical augmentation using a three-dimensional printed TCP/HA bone graft: A pilot study in dog mandible. *Clinical implant dentistry and related research* 18(6) (2016) 1183–1192.
78. J.-P. Carrel, A. Wiskott, M. Moussa, P. Rieder, S. Scherrer, S. Durual, A 3D printed TCP/HA structure as a new osteoconductive scaffold for vertical bone augmentation, *Clinical Oral Implants Research* 27 (2016) 55–62.
79. M. Moussa, J.P. Carrel, S. Scherrer, M. Cattani-Lorente, A. Wiskott, S. Durual, Medium-term function of a 3D printed TCP/HA structure as a new osteoconductive scaffold for vertical bone augmentation: A simulation by BMP-2 activation, *Materials* 8(5) (2015) 2174–2190.



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Chapter 13 Bioprinting of liver

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13.1 Introduction: Liver—a vital organ

The liver is one of the largest organs in the human body, accounting for approximately 2%–3% of the weight of an adult, and it resides between the organs of the gastrointestinal tract and the heart [1]. It performs numerous major functions such as detoxifying harmful substances, balancing the biochemical environment, synthesizing blood proteins, and metabolizing glucose and lipids [2]. Due to its multiple vital roles, the liver is considered as one of the most important organs in the human body.

The liver comprises up to 1.5 million hepatic lobules, which are its structural and functional units [3]. Each lobule has an approximately hexagonal cross-section with central veins surrounded by sinusoids [4]. Sinusoids are irregular capillaries composed of a layer of fenestrated endothelial cells. These endothelial cells form an endothelium that filters incoming blood from the portal vein and provides hepatocytes with oxygen and nutrients. The endothelium is separated from the hepatocytes by the space of Disse. The hexagonal structure of the liver lobule is required for maintaining liver functions: The hexagonal architecture enlarges the total contact area at the tissue level and enables rapid mass transfer from blood in the sinusoid into the hepatocytes for detoxification or metabolic purposes [5].

The liver exhibits a high regenerative capacity and continues to function even when a large portion is removed. However, in the case of end-stage liver failure, liver transplantation is the only effective therapy; however, transplantation is limited by the lack of liver donors [6]. To overcome this problem, numerous liver tissue-engineering studies have focused on the development of transplantable artificial livers. Furthermore, many candidates for new drugs have been withdrawn or rejected during drug development because they cause drug-induced liver injury or unexpected biotransformation in the liver. Therefore, there is also a need for *in vitro* liver models for pharmaceutical research to predict the pharmacokinetics–pharmacodynamics of novel chemicals.

Three-dimensional (3D) bioprinting is an innovative technology in liver tissue engineering that enables the preparation of complex 3D liver structures. Using bioprinting technology, multiple biomaterials and cell types can be delivered and positioned in a predetermined area. Recent studies on bioprinting technology have attempted to fabricate liver constructs for transplantation and pharmaceutical research. Here we give a comprehensive overview of liver bioprinting and present the cell sources, biomaterials, and bioprinting methods' key to this technique.

13.2 Cell sources for liver bioprinting

The liver is composed of many different types of cell with which its functions are tightly correlated. Hepatocytes, parenchymal cells of the liver, are responsible for major hepatic functions; therefore, they are essential components of functional liver constructs. Nonparenchymal cells, such as Kupffer cells, hepatic stellate cells (HSCs), cholangiocytes, and endothelial cells, are also required to develop a fully functional liver tissue because of their specific biochemical responsibilities and parenchyma-supporting functions. In this section, we introduce currently utilized liver-cell sources.

13.2.1 Parenchymal cells

The selection of suitable sources of liver parenchymal cells for liver tissue engineering is challenging. Currently, there are four possible sources of hepatocytes: (1) primary hepatocytes, (2) liver cancer-derived immortalized hepatocytes (LCH), (3) artificially immortalized hepatocytes, and (4) induced hepatocyte-like cells (Table 13.1).

Primary hepatocytes are the most effective cell source for the preparation of liver constructs because they exhibit the highest potency to reproduce native liver functions [7]. Primary hepatocytes from humans [8], rats [9], and mice [10] have been utilized in liver bioprinting. However, primary hepatocytes lose their phenotype and viability rapidly *in vitro* [11]. Moreover, it is difficult to isolate sufficient healthy hepatocytes from patients to generate liver constructs. Therefore, utilizing primary hepatocytes is challenging in terms of their isolation, maintenance [12], and proliferation [13]. To date, primary hepatocytes have only been used for small-scale *in vitro* liver models [14] and one-shot transplantable cell constructs in small animal models [15].

Table 13.1 Overview of Parenchymal Cell Sources for Liver Bioprinting

Parenchymal Cell Sources	Types	<i>In vitro</i> Expansion	Advantages	Disadvantages
Primary cultured hepatocytes	Human Rat Mouse	None or scarce	Recapitulation of <i>in vivo</i> hepatocyte functions	Difficult to isolate Difficult <i>in vitro</i> maintenance and expansion
Liver cancer-derived immortalized hepatocytes	Hepatoblastoma Hepatocellular carcinoma	Fast	Easy to utilize Unlimited proliferation	Not clinically applicable Defective functions
Artificially immortalized hepatocytes	Immortalized Conditionally immortalized	Fast or controlled expansion	Easy to supply Controllable expansion or maturation	Not clinically applicable
Induced hepatocyte-like cells	Pluripotency (iPSCs/ESCs)	Fast (before differentiation)	Matured hepatocyte-like cells	Ethical issues (ESCs) or teratoma formation (both) Defective functions
	Multipotency (BMMSCs/ASCs/AMSCs)		Stable phenotype Immunomodulatory functions	

LCHs are the most widely used source of liver parenchymal cells. Currently, established human LCH cell lines include HepG2 [16], Hep3b [17], Huh7 [18], SK-Hep-1 [19], and their derivatives such as C3A [20]. Consistent with their origin, the *in vitro* expansion and application of these cells are much easier than those of other sources of hepatocytes. LCHs are naturally immortalized hepatocytes but they exhibit defects compared with normal hepatocytes, such as low drug sensitivity [21] and reduced biotransformation activity [22]. Although a hepatic progenitor cell line, HepaRG, has been developed that demonstrates more mature hepatic functions [23], its applications are limited to *in vitro* drug screening or research into drug toxicity because of its liver-cancer origin.

Artificially immortalized hepatocytes are another source of parenchymal cells for liver bioprinting that maintain critical liver parenchymal functions with an indefinite proliferation profile [24]. Viral oncogenes, such as simian virus 40 large T antigen (SV40 Tag) [25], human papillomavirus Type 16 E6/E7 [26], and human telomerase reverse transcriptase [26], are transfected into normal hepatocytes to establish immortalized hepatocyte cell lines. These genes are inserted by various gene transfer methods such as plasmid transfer, viral infection [27], human artificial chromosomes [28], surmounting cell-cycle arrest [29], and telomere-dependent senescence [30]. Furthermore, with the temperature-sensitive SV40 Tag, the proliferation of hepatocytes can be controlled depending on the temperature [31], typically, at 33°C for proliferation and 37°C for maturation. To date, no reports have described the usage of artificially immortalized hepatocytes for bioprinting because their creation involves a complex process of genetic modification. However, artificially immortalized hepatocytes may represent a future source of parenchymal cells for liver bioprinting.

Hepatocyte-like cells induced from stem cells or hepatic progenitor cells are considered a promising cell source because they support the concept of personalized liver constructs for transplantation or drug-screening platforms. Moreover, because stem cells exhibit self-renewal and proliferation, sufficient cell numbers can be obtained for liver bioprinting after *in vitro* expansion. As sources of pluripotent stem cells (PSCs), embryonic stem cells (ESCs) [32] and induced-pluripotent stem cells (iPSCs) [33] have been applied in bioprinting; however, challenges related to the use of these PSCs remain, such as ethical issues (for ESCs) [7] and teratoma formation [34]. Multipotent adult stem cells, such as mesenchymal stem cells derived from bone marrow, have been utilized for the fabrication of bioprinted liver structures [35], adipose tissue [36], bone marrow [37], and amnion [38]. Although these multipotent stem cells demonstrate lower functionality and proliferation capabilities than PSCs, their stable phenotype,

low tumorigenesis, and immunomodulatory abilities may be beneficial for potential clinical applications [39]. To achieve hepatic differentiation of these stem cells, a combination of soluble factors, including hepatocyte growth factor, epidermal growth factor, fibroblast growth factor 4, oncostatin M, dexamethasone, and some bile-acid components, is necessary [40].

13.2.2 Nonparenchymal cells

Hepatic sinusoidal endothelial cells (HSEC) are liver-specific blood-vessel cells. Maintaining a bioprinted liver construct requires well-established oxygen and nutrient diffusion into potential regions of hypoxia [41]. Therefore, many studies have focused on vascularization using HSECs [42] or other kinds of endothelial cells, such as human umbilical vein endothelial cells (HUVECs) [43]. Other than their role in blood vessels, HSECs also support liver functions [44] by forming fenestrated blood vessels for macromolecular exchange in the liver and synthesizing Factor VIII [45].

Kupffer cells are a type of macrophage that inhabits the liver [46]. Their main responsibility is to protect the liver from pathologic conditions including infection, fibrosis, and liver cancer [47]. Moreover, they interact with hepatocytes and other nonparenchymal cells to support lipid metabolism and normal liver functions [48]. Primary Kupffer cells [49] and several established cell lines [50] are available, but only primary Kupffer cells have been used for liver bioprinting [42].

HSCs, also known as perisinusoidal or Ito cells, are star-shaped cells found primarily in the space of Disse in hepatic sinusoids. HSCs reportedly play critical roles in liver regeneration [51], liver morphogenesis [52], and the development of fibrosis [53] and cirrhosis [54]; therefore, their inclusion in liver constructs is important. Regarding the source of HSCs, primary cultured HSCs [55] and immortalized HSCs, such as LX1 [56], LX2 [57], and TWNT-1 [58], are available. Among them, the use of primary HSCs for liver bioprinting has been reported [42].

Cholangiocytes are epithelial cells that form the biliary tree, a tubular network for the transport of bile acids [59]. In native liver tissue, hepatocytes synthesize bile-acid compounds and secrete them into the bile canaliculi, a process organized by the specific polarity of hepatocytes [5]. Then, bile acids are transported to the biliary network, where they are modified by cholangiocytes and finally secreted as a digestive juice [60]. Although cholangiocyte sources including primary cultured cells [61] and artificially immortalized cell lines [62] have been established, their application in liver bioprinting has not been reported to date ([Table 13.2](#)).

Table 13.2 Overview of Nonparenchymal Cell Sources for Liver Bioprinting

Nonparenchymal Cells	Types	Functions	Source
Hepatic sinusoidal endothelial cells	Endothelial cells	Fenestrated blood-vessel formation Factor VIII synthesis	Isolation Commercialized primary cultured cells TMNK-1 and TRP3 cell lines
Kupffer cells	Macrophages	Immune or inflammatory response Hepatic pathology regulation	Isolation Commercialized primary cultured cells RKC1, RKC2, and KUP5 cell lines
Hepatic stellate cells	Hepatic interstitial cells	Fat storage Liver regeneration Liver morphogenesis Hepatic pathology regulation	Isolation LX1, LX2 Commercialized primary cultured cells LX1, LX2, TWNT-1, and LI90 cell lines
Cholangiocytes	Biliary epithelial cells	Bile duct formation Bile-acid component regulation	Isolation Commercialized primary cultured cells H69 and NHC cell lines

13.3 Biomaterials for liver bioprinting

There are multiple candidate biomaterials for liver bioprinting including natural and synthetic polymers. Selection of the appropriate biomaterials is important for generating a functional liver construct. Therefore, numerous studies have evaluated biomaterials for liver tissue engineering. In this section, we discuss these biomaterials in detail ([Table 13.3](#)).

13.3.1 Natural polymers

To fabricate a cell-laden liver construct via bioprinting, the properties of the bio-material are critical. Biocompatibility is indispensable, and the biomaterial also requires mechanical properties suitable for maintaining the shape of bioprinted constructs and adequate biochemical functionality for the survival and function of liver cells. Unmodified natural polymers can be applied alone as a bioink, but they can also be chemically modified to improve their mechanical strength, bioactivity, or specific functionality. In this section, natural polymers and their derivatives for liver bioprinting are introduced.

Table 13.3 Overview of Biomaterials for Liver Bioprinting

Biomaterials		Cross-Linking Methods	Cross-Linking Rate	Functional Modification
Raw biomaterial	Collagen	Thermal cross-linking (~37°C) Aldehyde compounds (glutaraldehyde, glyceraldehyde, etc.) Genipin	Slow	Methacrylation (photocurability) Galactosylation
	Gelatin	Aldehyde compounds (glutaraldehyde, glyceraldehyde, etc.) Genipin Tyrosinase	Slow	Methacrylation (photocurability) Galactosylation
	Alginate	Calcium chloride	Fast	Cell binding-motif conjugation (RGD, DGEA, and GFOGER) Galactosylation
ECM complex	Matrigel® Decellularized liver-derived ECM	Thermal cross-linking (~37°C)	Slow	
Synthetic materials	PCL, PLA, PGA, PLGA, and PDMS			

13.3.2 Collagen and its derivatives

Collagen is the most abundant structural protein in the body and a commonly used biomaterial in stem cell culture [63], hepatocyte maintenance [64], scaffolds [65], and printed liver constructs [43]. Many types of commercialized collagen are available from sources such as humans, pigs, goats, rabbits, rats, and mice; type I collagen is the most commonly used. Collagen is in solution form at low temperatures (around 4°C) and irreversibly cross-linked at physiologic temperatures (around 37°C). In addition to this thermally sensitive gelation, it can also be cross-linked by chemicals such as glyceraldehyde [66]; however, these cross-linking agents can have detrimental effects on cells and the implanted site. Riboflavin (vitamin B2) has been utilized as a cross-linking agent for collagen in ultraviolet (UV) A-induced photocross-linking [67]. This method is less toxic than direct cross-linking with aldehyde components [68]. Moreover, the mechanical properties of collagen constructs are enhanced by UVA-induced photocross-linking; however, UVA is harmful to cells [69]. Collagen also can be modified

with methacrylate for rapid cross-linking [70]. Using UV light and a photoinitiator, methacrylated collagen undergoes rapid cross-linking in tens of seconds and exhibits enhanced mechanical properties. Galactosylation is another option for the functional modification of collagen [71]. The asialoglycoprotein receptors of hepatocytes recognize galactosyl groups in the environment, and the receptor–ligand interaction enhances the attachment and functionality of the hepatocytes [71]. Therefore, the conjugation of galactosyl groups has been studied in collagen and other hydrogels for the improvement of hepatic functionality [72]. Although the functional modification of collagen has not been utilized for liver bioprinting, this technique may have applications in the development of functional liver tissue.

13.3.3 Gelatin and its derivatives

Gelatin is an irreversibly denatured collagen that has thermoreversible gelation properties: It becomes a gel at temperatures below 4°C and a liquid at temperatures above 37°C [43]. Gelatin has been utilized as an *in vitro* substrate for purposes such as cell expansion [73], maintenance [74], and stem cell differentiation [75] in many studies. Due to its gelation properties, gelatin cannot be cross-linked under physiologic conditions (around 37°C). Therefore, additional cross-linking is necessary for the fabrication of cell-laden structures by bioprinting. Reportedly, glutaraldehyde [76], genipin [77], and tyrosinase [78] act as cross-linking agents for gelatin. Gelatin mixtures with other polymers, such as chitosan [79] and alginate [80], can also be utilized for maintaining bioprinted liver structures. Gelatin methacrylate (GelMA), gelatin chemically conjugated with methacrylate [81], has been widely utilized for the bioprinting of liver [82] and other tissues [83]. However, GelMA is cross-linked by UV light under photoinitiator activation [84], and some photoinitiators, such as 2,2-dimethoxy-2-phenylacetophenone or Irgacure 2959, are toxic [85]. Therefore, photoinitiator concentration and UV-exposure time should be considered when preparing material for bioprinting.

13.3.4 Alginate and its derivatives

Derived from brown algae, alginate is an anionic polysaccharide comprising repeated mannuronic and guluronic acids [86]. In the presence of calcium ions, which are usually supplied by calcium chloride solution, alginate solution immediately forms a hydrogel through ionic cross-linking, and its mechanical properties change to maintain its shape [87]. Due to its biocompatibility, rapid cross-linking, and inexpensiveness, it is the most widely used biomaterial in liver bioprinting [80]. However, the composition of native alginate does not provide regions for cell attachment; therefore, adherent cells cannot fully recapitulate their normal behavior [88]. To overcome this bioinert property, the RGD (Arg-Gly-Asp) peptide, a cell-binding region present in Fibronectin and Laminin, was conjugated with alginate to enable the fabrication of bioactive alginate constructs via

bioprinting [32]. Collagen-derived DGEA (Asp-Gly-Glu-Ala) [89] and GFOGER (Gly-Phe-Hyp-Gly-Glu-Arg) [90] have been also conjugated with alginate to yield bioactive alginate environments.

13.3.5 Matrigel®

Matrigel® is a commercially available hydrogel derived from murine sarcoma cells with thermal gelation properties equal to those of collagen [91]. Because of its origin, Matrigel provides cells with a more suitable microenvironment than the aforementioned materials. Many reports describe culture methods that involve Matrigel to maintain the phenotype [92] or improve functionality [93] of primary hepatocytes. Moreover, Matrigel has also been used as a biomaterial to fabricate HepG2-laden constructs [94]. Although Matrigel provides an outstanding micro-environment, it cannot fully support tissue-specific growth factors or cytokines. Furthermore, the heterogeneous qualities of Matrigel can result in variable cellular responses, and the tumor origin of Matrigel inhibits clinical applications in humans because of its tumorigenic potential [95]. Nevertheless, Matrigel is considered as one of the most effective biomaterials for liver bioprinting.

13.3.6 Decellularized liver extracellular matrix

Decellularized liver extracellular matrix (ECM) is a promising biomaterial for liver tissue engineering and bioprinting because it contains liver-specific biochemical components, including glycosaminoglycan, soluble factors, many types of collagen, and other structural proteins of the native liver. Whole-liver decellularized tissue repopulated with liver cells demonstrated potential for the creation of transplantable liver [15,96,97]. Decellularized liver ECM can be solubilized by enzymes and acids and can be utilized as a hydrogel material after the neutralization of its physiologic pH. Decellularized liver ECM can be applied as a substrate for two-dimensional (2D) culture [98,99], as a scaffold for 3D culture [100], and as a bioink for bioprinting [35,42]. In addition, decellularized liver ECM can be manipulated by the inclusion of additives such as photocurable modified gelatin to match the mechanical properties of native liver [42].

13.3.7 Synthetic polymers

Synthetic polymers are useful for adjusting mechanical properties in various biomedical applications. Synthetic polymers can be utilized as scaffolds or structural frameworks to support materials for fabricating biologic constructs using bioprinting. Various synthetic polymers can be used in bioprinting; among them, polycaprolactone, polylactic acid, polyglycolic acid, and polylactic-co-glycolic acid are most commonly used for 3D bioprinting. The biodegradability and biocompatibility of these materials [101] enable their application in bioprinting, and

their thermal manipulability is a critical advantage [102]. However, these materials are often too stiff for liver bioprinting because liver is one of the softest tissues in the human body.

13.4 Methods of liver bioprinting

Various bioprinting techniques have been developed that differ in the method of dispensing biomaterials and cells. Many of these bioprinting methods have been used to create liver organoids or *in vitro* screening models. Each printing method has pros and cons for the preparation of liver constructs. In this section, we briefly introduce each bioprinting technique and its application in liver bioprinting.

13.4.1 Inkjet bioprinting

The inkjet bioprinting method manages droplet from a cell-suspended liquid. The droplets are generated by electrically heating the nozzle to induce a vapor bubble [103] or breaking the liquid into droplets using a piezoelectric actuator [104,105]. The inkjet printing method enables to control the small volume of cell-suspended liquid and it is possible to get highest resolutions up to 20 μm [106]. Therefore, inkjet bioprinting method has a high potential for fabricating the precise and microscale tissues in the narrow area. However, it is hard to fabricate the biological structure in large scale. In this manner, with inkjet bioprinting technology, only liver cell array for the drug screening platform has been introduced [107]. Due to the advancement of inkjet bioprinting, they have printed HepG2 cells and HUVEC cells in the layer-by-layer arrangement. Similar to another cocultured method, this study also demonstrated that coprinting of each cell and layer-by-layer arrangement of cells has enhanced the liver functions.

13.4.2 Microextrusion bioprinting

Microextrusion is the most common method used to print complex biologic structures. Printed materials are deposited on to a substrate by a microextrusion head. Using this technique, biomaterials and cells can be selectively dispensed at an intended location through nozzles and needles using physical forces. Pneumatic [78,108] or mechanical [109–111] pressure generated using a piston is an example of the forces used to drive microextrusion printing. A microextrusion-based system equipped with multiple printing heads containing different cells/biomaterials can be used to prepare complex, heterogeneous structures [112]. Numerous studies of liver bioprinting using microextrusion printing systems have been conducted. Kim et al. [10] demonstrated bioprinting of mouse primary hepatocytes using alginate biomaterial. They determined that bioprinting can be used for the long-term culture of mouse primary hepatocytes. Faulkner-Jones et al. [32] generated

a bioprinted miniliver using human stem cells with alginate. The hepatic differentiation of the printed structure was analyzed, and the authors claimed that patient-specific models can be generated by this bioprinting method. Some studies have used both parenchymal and nonparenchymal cells to improve functionality. Lee et al. [108] used primary hepatocytes, human lung fibroblasts, and HUVECs to print liver tissue that exhibited enhanced liver-specific functions of urea and albumin secretion (Figure 13.1a).

A *liver-on-a-chip* has also been developed for drug screening. To develop the liver-on-a-chip, HepG2 cells were printed on to a prepared platform.

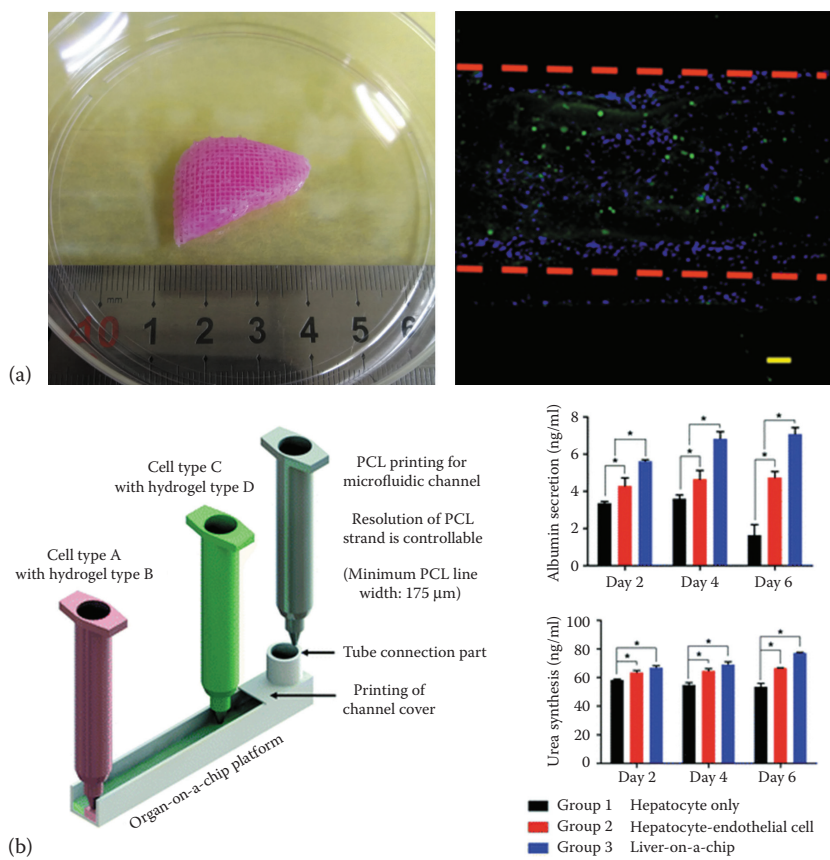


Figure 13.1 Three-dimensional (3D)-bioprinted liver tissues. (a) 3D-bioprinted liver tissue and albumin secretion by the tissue. (b) Schematic diagram of the fabrication of a 3D-bioprinted liver-on-a-chip and metabolite secretion by the liver-on-a-chip. (Reproduced with permission [43, 108].)

Microextrusion printing was first used to create the liver-on-a-chip platform [113,114]. Subsequently, hepatic spheroids comprising HepG2/C3A cells were printed on to the prepared chip platform for liver-on-a-chip development. As hepatocytes are sensitive (they lose their characteristics and viability rapidly), this rapid and simple fabrication method is suitable for retaining hepatic functionality. Recently, Lee et al. [43] developed a liver-on-a-chip platform with a one-step fabrication method using a polymer, HepG2 cells, and HUVECs. These three different materials were used to print a single construct via the microextrusion printing method. The construct showed enhanced liver functions as a result of the coculture of liver cells (Figure 13.1b). Although there are multiple examples of printed liver models, further studies are necessary to develop fully functional liver models via microextrusion printing.

13.4.3 Laser-assisted bioprinting

Laser-assisted bioprinting yields high-resolution printed biologic structures. The laser-guided direct-writing method traps cells in a laser beam using the difference in refractive index between cells and medium [19]. The trapped cells are then pushed axially through a layer of a low-viscosity material to a hydrogel-coated surface. Laser-induced cell-printing systems produce droplets of cells by focusing laser pulses on a cell-laden, hydrogel-coated transparent support called a ribbon [115,116]. A laser-induced vapor pocket forms and the droplet separates from the ribbon and transfers to the receiving substrate. Laser-assisted bioprinting can overcome some of the limitations of microextrusion and inkjet bioprinting, such as their lack of precision regarding the shape of the microscale structure. Recently, Ma et al. [33] developed a patterned biomimetic human iPSC-derived *in vitro* liver model using laser-assisted bioprinting. It mimicked the liver lobule structure, which is difficult to fabricate using microextrusion or inkjet bioprinting. Furthermore, they used iPSC-derived hepatocytes, HUVECs, and adipose-derived stem cells to develop the model, and the cells were aligned in a biomimetic manner.

13.6 Conclusion and future perspectives

Significant progress has been made in the development of artificial liver tissues using bioprinting technology. 3D bioprinting facilitates the generation of artificial liver tissue by directly dispensing cells in a predetermined area with spatiotemporal control. Various 3D-bioprinting techniques have been used for the fabrication of liver tissue, including inkjet, microextrusion and laser-assisted bioprinting, that allows the creation of complex, heterogeneous constructs with hepatic functionality. Although some hepatic functions have been replicated in these bioprinted tissues, fabrication of fully functional liver tissue remains at an early stage. To date, only a few parenchymal and nonparenchymal cell types have

been utilized to generate bioprinted liver tissue. As nonparenchymal cells reportedly enhance hepatic functionality strongly, the inclusion of all types of liver cell, including hepatocytes, Kupffer cells, endothelial cells, stellate cells, and cholangiocytes, may recapitulate the functionality of the liver. Patient-derived iPSCs can also contribute to the bioprinting of fully functional liver tissue because they are able to proliferate and differentiate into hepatic progenitor cells; sufficient parenchymal cells can be achieved for bioprinting. Moreover, bioprinted *in vitro* liver models or bioprinted liver chips that exhibit patient-specific pathologic phenotypes can be produced by employing iPSCs.

Hydrogels, also called bioinks, must exhibit suitable viscoelasticity to maintain the shape of constructs after bioprinting and to provide cells with a microenvironment that enhances cell adhesion, growth, and differentiation. In this regard, liver tissue-specific bioinks that possess suitable printability, a similar composition, and microenvironment to those of the native liver require further investigation. Finally, methods of achieving a more precise spatiotemporal resolution should be developed to reproduce the complex architecture of the liver, particularly the hexagonal liver lobules that enable the rapid mass transfer of nutrients, oxygen, and metabolites. Despite current challenges, the process of bioprinting liver tissue continues to evolve and the development of fully functional transplantable liver tissues can be expected in the near future.

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References

1. Abdel-Misih SR, Bloomston M. Liver anatomy. *Surgical Clinics of North America* 2010;90:643–653.
2. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nature Medicine* 2003;9:213–219.
3. Materne E-M, Tonevitsky AG, Marx U. Chip-based liver equivalents for toxicity testing—organotypicalness versus cost-efficient high throughput. *Lab on a Chip* 2013;13:3481–3495.
4. Yamaguchi T, Hachiya H, Kamiyama N. Modeling of the cirrhotic liver considering the liver lobule structure. *Japanese Journal of Applied Physics* 1999;38:3388–3392.
5. Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S et al. Recent advances in 2D and 3D *in vitro* systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of Toxicology* 2013;87:1315–1530.

6. Brown RS. Live donors in liver transplantation. *Gastroenterology* 2008;134:1802–1813.
7. Palakkan AA, Hay DC, Kumary TV, Ross JA. Liver tissue engineering and cell sources: Issues and challenges. *Liver International* 2013;33:666–676.
8. Zhong C, Xie H-Y, Zhou L, Xu X, Zheng S-S. Human hepatocytes loaded in 3D bio-printing generate mini-liver. *Hepatobiliary & Pancreatic Diseases International* 2016;15:512–518.
9. Miller JS, Stevens KR, Yang MT, Baker BM, Nguyen D-HT, Cohen DM et al. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials* 2012;11:768–774.
10. Kim Y, Kang K, Jeong J, Paik SS, Kim JS, Park SA et al. Three-dimensional (3D) printing of mouse primary hepatocytes to generate 3D hepatic structure. *Annals of Surgical Treatment and Research* 2017;92:67–72.
11. Hewitt NJ. Optimisation of the cryopreservation of primary hepatocytes. *Hepatocytes: Methods and Protocols* 2010:83–105.
12. Gerlach JC, Schnoy N, Encke J, Smith MD, Müller C, Neuhaus P. Improved hepatocyte in vitro maintenance in a culture model with woven multicompartiment capillary systems: Electron microscopy studies. *Hepatology* 1995;22:546–552.
13. Cho CH, Berthiaume F, Tilles AW, Yarmush ML. A new technique for primary hepatocyte expansion in vitro. *Biotechnology and Bioengineering* 2008;101:345–356.
14. Guguen-Guillouzo C, Guillouzo A. General review on in vitro hepatocyte models and their applications. *Hepatocytes: Methods and Protocols* 2010:1–40.
15. Zhou P, Lessa N, Estrada DC, Severson EB, Lingala S, Zern MA et al. Decellularized liver matrix as a carrier for the transplantation of human fetal and primary hepatocytes in mice. *Liver Transplantation* 2011;17:418–427.
16. Yoshitomi S, Ikemoto K, Takahashi J, Miki H, Namba M, Asahi S. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. *Toxicology in Vitro* 2001;15:245–256.
17. Zhu XH, Gan SK, Wang CH, Tong YW. Proteins combination on PHBV microsphere scaffold to regulate Hep3B cells activity and functionality: A model of liver tissue engineering system. *Journal of Biomedical Materials Research Part A* 2007;83:606–616.
18. Barrila J, Radtke AL, Crabbé A, Sarker SF, Herbst-Kralovetz MM, Ott CM et al. Organotypic 3D cell culture models: Using the rotating wall vessel to study host–pathogen interactions. *Nature Reviews Microbiology* 2010;8:791–801.
19. Nahmias Y, Schwartz RE, Verfaillie CM, Odde DJ. Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnology and Bioengineering* 2005;92:129–136.
20. Elkayam T, Amitay-Shaprut S, Dvir-Ginzberg M, Harel T, Cohen S. Enhancing the drug metabolism activities of C3A—a human hepatocyte cell line—by tissue engineering within alginate scaffolds. *Tissue Engineering* 2006;12:1357–1368.
21. Gerets H, Tilmant K, Gerin B, Chanteux H, Depelchin B, Dhalluin S et al. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biology and Toxicology* 2012;28:69–87.
22. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metabolism and Disposition* 2003;31:1035–1042.
23. Kanebratt KP, Andersson TB. Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metabolism and Disposition* 2008;36:1444–1452.
24. Ramboer E, De Craene B, De Kock J, Vanhaecke T, Berx G, Rogiers V et al. Strategies for immortalization of primary hepatocytes. *Journal of Hepatology* 2014;61:925–943.

25. Smalley M, Leiper K, Tootle R, McCloskey P, O'Hare M, Hodgson H. immortalization of human hepatocytes by temperature-sensitive SV40 large-T antigen. *In Vitro Cellular & Developmental Biology-Animal* 2001;37:166–1668.
26. Tsuruga Y, Kiyono T, Matsushita M, Takahashi T, Kasai N, Matsumoto S et al. Effect of intrasplenic transplantation of immortalized human hepatocytes in the treatment of acetaminophen-induced acute liver failure SCID mice. *Transplantation Proceedings* 2008;40:617–619.
27. Wang X, Mani P, Sarkar DP, Roy-Chowdhury N, Roy-Chowdhury J. Ex vivo gene transfer into hepatocytes. *Hepatocyte Transplantation: Methods and Protocols* 2009:117–139.
28. Ito M, Ito R, Yoshihara D, Ikeno M, Kamiya M, Suzuki N et al. Immortalized hepatocytes using human artificial chromosome. *Cell Transplantation* 2008;17:165–171.
29. Maruyama M, Kobayashi N, Westerman KA, Sakaguchi M, Allain JE, Totsugawa T et al. Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT. *Transplantation* 2004;77:446–451.
30. Wege H, Chui MS, Le HT, Strom SC, Zern MA. In vitro expansion of human hepatocytes is restricted by telomere-dependent replicative aging. *Cell Transplantation* 2003;12:897–906.
31. Dong SH, Kim BH, Nam GD, Jang JY, Kim NH, Lee SK et al. Immortalized hepatocytes established with a temperature-sensitive SV40 T antigen could be independent of T antigen. *Korean Journal of Medicine* 2005;68:268–276.
32. Faulkner-Jones A, Fyfe C, Cornelissen D-J, Gardner J, King J, Courtney A et al. Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. *Biofabrication* 2015;7:044102.
33. Ma X, Qu X, Zhu W, Li Y-S, Yuan S, Zhang H et al. Deterministically patterned biomimetic human iPSC-derived hepatic model via rapid 3D bioprinting. *Proceedings of the National Academy of Sciences of the United States of America* 2016;113:2206–2211.
34. Zhang WY, de Almeida PE, Wu JC. Teratoma formation: A tool for monitoring pluripotency in stem cell research. 2012.
35. Lee H, Han W, Kim H, Ha D-H, Jang J, Kim B-S, Cho D-W. Development of liver decellularized extracellular matrix bioink for 3D cell printing-based liver tissue engineering. *Biomacromolecules* 2017;18:1229–1237.
36. Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Quinn G et al. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* 2007;46:219–228.
37. Ishii K, Yoshida Y, Akechi Y, Sakabe T, Nishio R, Ikeda R et al. Hepatic differentiation of human bone marrow-derived mesenchymal stem cells by tetracycline-regulated hepatocyte nuclear factor 3 β . *Hepatology* 2008;48:597–606.
38. Lindenmair A, Hatlapatka T, Kollwig G, Hennerbichler S, Gabriel C, Wolbank S et al. Mesenchymal stem or stromal cells from amnion and umbilical cord tissue and their potential for clinical applications. *Cells* 2012;1:1061–1088.
39. Liao S-Y, Tse H-F. Multipotent (adult) and pluripotent stem cells for heart regeneration: What are the pros and cons? *Stem Cell Research & Therapy* 2013;4:151.
40. Sawitza I, Kordes C, Götze S, Herebian D, Häussinger D. Bile acids induce hepatic differentiation of mesenchymal stem cells. *Scientific Reports* 2015;5:13320.
41. Li J, Chen M, Fan X, Zhou H. Recent advances in bioprinting techniques: Approaches, applications and future prospects. *Journal of Translational Medicine* 2016;14:271.
42. Skardal A, Devarasetty M, Kang H-W, Mead I, Bishop C, Shupe T et al. A hydrogel bioink toolkit for mimicking native tissue biochemical and mechanical properties in bioprinted tissue constructs. *Acta Biomaterialia* 2015;25:24–34.
43. Lee H, Cho D-W. One-step fabrication of an organ-on-a-chip with spatial heterogeneity using a 3D bioprinting technology. *Lab on a Chip* 2016;16:2618–2625.

44. DeLeve LD. Liver sinusoidal endothelial cells and liver regeneration. *The Journal of Clinical Investigation* 2013;123:1861–1866.
45. Shahani T, Covens K, Lavend'homme R, Jazouli N, Sokal E, Peerlinck K et al. Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. *Journal of Thrombosis and Haemostasis* 2014;12:36–42.
46. Zinchenko YS, Schrum LW, Clemens M, Coger RN. Hepatocyte and kupffer cells co-cultured on micropatterned surfaces to optimize hepatocyte function. *Tissue Engineering* 2006;12:751–761.
47. Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. Kupffer cells in the liver. *Comprehensive Physiology* 2013;3:785–797.
48. Nguyen-Lefebvre AT, Horuzsko A. Kupffer cell metabolism and function. *Journal of Enzymology and Metabolism* 2015;1:101.
49. Olynyk JK, Clarke SL. Isolation and primary culture of rat Kupffer cells. *Journal of Gastroenterology and Hepatology* 1998;13:843–845.
50. Kitani H, Sakuma C, Takenouchi T, Sato M, Yoshioka M, Yamanaka N. Establishment of c-myc-immortalized Kupffer cell line from a C57BL/6 mouse strain. *Results in Immunology* 2014;4:68–74.
51. Kordes C, Sawitzka I, Götze S, Herebian D, Häussinger D. Hepatic stellate cells contribute to progenitor cells and liver regeneration. *The Journal of Clinical Investigation* 2014;124:5503–5515.
52. Asahina K. Hepatic stellate cell progenitor cells. *Journal of Gastroenterology and Hepatology* 2012;27:80–84.
53. Moreira RK. Hepatic stellate cells and liver fibrosis. *Archives of Pathology & Laboratory Medicine* 2007;131:1728–1734.
54. Brandão DF, Ramalho LN, Ramalho FS, Zucoloto S, Martinelli AdLC, Castro e Silva Od. Liver cirrhosis and hepatic stellate cells. *Acta Cirurgica Brasileira* 2006;21:54–57.
55. Weiskirchen R, Gressner AM. Isolation and culture of hepatic stellate cells. *Fibrosis Research: Methods and Protocols* 2005:99–113.
56. Xu L, Hui A, Albanis E, Arthur M, O'Byrne S, Blaner W et al. Human hepatic stellate cell lines, LX-1 and LX-2: New tools for analysis of hepatic fibrosis. *Gut* 2005;54:142–151.
57. Weiskirchen R, Weimer J, Meurer SK, Kron A, Seipel B, Vater I et al. Genetic characteristics of the human hepatic stellate cell line LX-2. *PloS One* 2013;8:e75692.
58. Chen AA, Thomas DK, Ong LL, Schwartz RE, Golub TR, Bhatia SN. Humanized mice with ectopic artificial liver tissues. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108:11842–11847.
59. Tietz PS, LaRusso NF. Cholangiocyte biology. *Current Opinion in Gastroenterology* 2006;22:279–287.
60. Tabibian JH, Masyuk AI, Masyuk TV, O'Hara SP, LaRusso NF. Physiology of cholangiocytes. *Comprehensive Physiology* 2013;3:541–565.
61. Tanimizu N, Nakamura Y, Ichinohe N, Mizuguchi T, Hirata K, Mitaka T. Hepatic biliary epithelial cells acquire epithelial integrity but lose plasticity to differentiate into hepatocytes in vitro during development. *Journal of Cell Science* 2013;126:5239–5246.
62. Tabibian JH, Trussone CE, O'Hara SP, Splinter PL, Heimbach JK, LaRusso NF. Characterization of cultured cholangiocytes isolated from livers of patients with primary sclerosing cholangitis. *Laboratory Investigation* 2014;94:1126–1133.
63. Leong WS, Tay CY, Yu H, Li A, Wu SC, Duc D-H et al. Thickness sensing of hMSCs on collagen gel directs stem cell fate. *Biochemical and Biophysical Research Communications* 2010;401:287–292.
64. Vinken M, Elaut G, Henkens T, Papeleu P, Snykers S, Vanhaecke T et al. Rat hepatocyte cultures: Collagen gel sandwich and immobilization cultures. *Cytochrome P450 Protocols* 2006:247–254.

65. Li J, Li L, Yu H, Cao H, Gao C, Gong Y. Growth and metabolism of human hepatocytes on biomodified collagen poly (lactic-co-glycolic acid) three-dimensional scaffold. *ASAIJ Journal* 2006;52:321–327.
66. Wollensak G, Iomdina E. Long-term biomechanical properties after collagen crosslinking of sclera using glyceraldehyde. *Acta Ophthalmologica* 2008;86:887–893.
67. Henriquez MA, Izquierdo Jr L, Bernilla C, Zakrzewski PA, Mannis M. Riboflavin/Ultraviolet A corneal collagen cross-linking for the treatment of keratoconus: Visual outcomes and Scheimpflug analysis. *Cornea* 2011;30:281–286.
68. Avila MY, Gerena VA, Navia JL. Corneal crosslinking with genipin, comparison with UV-riboflavin in ex-vivo model. *Molecular Vision* 2012;18:1068.
69. Kim M, Takaoka A, Hoang QV, Trokel SL, Paik DC. Pharmacologic alternatives to riboflavin photochemical corneal cross-linking: A comparison study of cell toxicity thresholds. *Investigative Ophthalmology & Visual Science* 2014;55:3247–3257.
70. Brinkman WT, Nagapudi K, Thomas BS, Chaikof EL. Photo-cross-linking of type I collagen gels in the presence of smooth muscle cells: Mechanical properties, cell viability, and function. *Biomacromolecules* 2003;4:890–895.
71. Ghodsizadeh A, Hosseinkhani H, Piryaei A, Pournasr B, Najarasl M, Hiraoka Y et al. Galactosylated collagen matrix enhanced in vitro maturation of human embryonic stem cell-derived hepatocyte-like cells. *Biotechnology Letters* 2014;36:1095–1106.
72. Gevaert E, Billiet T, Declercq H, Dubrue P, Cornelissen R. Galactose-functionalized gelatin hydrogels improve the functionality of encapsulated hep2 cells. *Macromolecular Bioscience* 2014;14:419–427.
73. Bahrami SB, Veis M, Boudreau NJ. Isolation and expansion of endothelial progenitor cells derived from mouse embryonic stem cells. *Progenitor Cells: Methods and Protocols* 2012:81–96.
74. Mohr JC, de Pablo JJ, Palecek SP. 3-D microwell culture of human embryonic stem cells. *Biomaterials* 2006;27:6032–6042.
75. Yamada T, Yoshikawa M, Kanda S, Kato Y, Nakajima Y, Ishizaka S et al. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. *Stem Cells* 2002;20:146–154.
76. Ai H, Mills DK, Jonathan AS, Jones SA. Gelatin–glutaraldehyde cross-linking on silicone rubber to increase endothelial cell adhesion and growth. *In Vitro Cellular & Developmental Biology-Animal* 2002;38:487–492.
77. Bigi A, Cojazzi G, Panzavolta S, Roveri N, Rubini K. Stabilization of gelatin films by crosslinking with genipin. *Biomaterials* 2002;23:4827–4832.
78. Das S, Pati F, Choi Y-J, Rijal G, Shim J-H, Kim SW et al. Bioprintable, cell-laden silk fibroin–gelatin hydrogel supporting multilineage differentiation of stem cells for fabrication of three-dimensional tissue constructs. *Acta Biomaterialia* 2015;11:233–246.
79. Zhang Y, Wang QS, Yan K, Qi Y, Wang GF, Cui YL. Preparation, characterization, and evaluation of genipin crosslinked chitosan/gelatin three-dimensional scaffolds for liver tissue engineering applications. *Journal of Biomedical Materials Research Part A* 2016;104:1863–1870.
80. Jeon H, Kang K, Park SA, Kim WD, Paik SS, Lee S-H et al. Generation of multi-layered 3D structures of HepG2 cells using a bio-printing technique. *Gut and Liver* 2017;11:121–128.
81. Van Den Bulcke AI, Bogdanov B, De Rooze N, Schacht EH, Cornelissen M, Berghmans H. Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules* 2000;1:31–38.
82. Bertassoni LE, Cardoso JC, Manoharan V, Cristino AL, Bhise NS, Araujo WA et al. Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels. *Biofabrication* 2014;6:024105.

83. Kolesky DB, Truby RL, Gladman A, Busbee TA, Homan KA, Lewis JA. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials* 2014;26:3124–3130.
84. Yue K, Trujillo-de Santiago G, Alvarez MM, Tamayol A, Annabi N, Khademhosseini A. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* 2015;73:254–271.
85. Xu L, Sheybani N, Yeudall WA, Yang H. The effect of photoinitiators on intracellular AKT signaling pathway in tissue engineering application. *Biomaterials Science* 2015;3:250–255.
86. Draget KI, Smidsrød O, Skjåk-Bræk G. Alginates from algae. *Biopolymers Online* 2005.
87. Kuo CK, Ma PX. Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate and mechanical properties. *Biomaterials* 2001;22:511–521.
88. Jia J, Richards DJ, Pollard S, Tan Y, Rodriguez J, Visconti RP et al. Engineering alginate as bioink for bioprinting. *Acta Biomaterialia* 2014;10:4323–4331.
89. Mehta M, Madl CM, Lee S, Duda GN, Mooney DJ. The collagen I mimetic peptide DGEA enhances an osteogenic phenotype in mesenchymal stem cells when presented from cell-encapsulating hydrogels. *Journal of Biomedical Materials Research Part A* 2015;103:3516–3525.
90. Stephan SB, Taber AM, Jileeva I, Pegues EP, Sentman CL, Stephan MT. Biopolymer implants enhance the efficacy of adoptive T-cell therapy. *Nature Biotechnology* 2015;33:97–101.
91. Mulyasmita W, Lee JS, Heilshorn SC. Molecular-level engineering of protein physical hydrogels for predictive sol–gel phase behavior. *Biomacromolecules* 2011;12:3406–411.
92. Bi Y-a, Kazolias D, Duignan DB. Use of cryopreserved human hepatocytes in sandwich culture to measure hepatobiliary transport. *Drug Metabolism and Disposition* 2006;34:1658–1665.
93. Haouzi D, Baghdiguian S, Granier G, Travo P, Mangeat P, Hibner U. Three-dimensional polarization sensitizes hepatocytes to Fas/CD95 apoptotic signalling. *Journal of Cell Science* 2005;118:2763–2773.
94. Snyder J, Hamid Q, Wang C, Chang R, Emami K, Wu H et al. Bioprinting cell-laden matrigel for radioprotection study of liver by pro-drug conversion in a dual-tissue microfluidic chip. *Biofabrication* 2011;3:034112.
95. Benton G, Kleinman HK, George J, Arnaoutova I. Multiple uses of basement membrane-like matrix (BME/Matrigel) in vitro and in vivo with cancer cells. *International Journal of Cancer* 2011;128:1751–1757.
96. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis M-L, Guzzardi MA, Shulman C et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nature Medicine* 2010;16:814–820.
97. Mazza G, Rombouts K, Hall AR, Urbani L, Luong TV, Al-Akkad W et al. Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. *Scientific Reports* 2015;5:13079.
98. Lee JS, Shin J, Park H-M, Kim Y-G, Kim B-G, Oh J-W et al. Liver extracellular matrix providing dual functions of two-dimensional substrate coating and three-dimensional injectable hydrogel platform for liver tissue engineering. *Biomacromolecules* 2013;15:206–218.
99. Sellaro TL, Ranade A, Faulk DM, McCabe GP, Dorko K, Badyrak SF et al. Maintenance of human hepatocyte function in vitro by liver-derived extracellular matrix gels. *Tissue Engineering Part A* 2009;16:1075–1082.

100. Lang R, Stern MM, Smith L, Liu Y, Bharadwaj S, Liu G et al. Three-dimensional culture of hepatocytes on porcine liver tissue-derived extracellular matrix. *Biomaterials* 2011;32:7042–7052.
101. O'Brien FJ. Biomaterials & scaffolds for tissue engineering. *Materials Today* 2011;14:88–95.
102. Do AV, Khorsand B, Geary SM, Salem AK. 3D printing of scaffolds for tissue regeneration applications. *Advanced Healthcare Materials* 2015;4:1742–1762.
103. Kador KE, Grogan SP, Dorthé EW, Venugopalan P, Malek MF, Goldberg JL et al. Control of retinal ganglion cell positioning and neurite growth: Combining 3D printing with radial electrospun scaffolds. *Tissue Engineering* 2016;22:286–294.
104. Cheng E, Yu H, Ahmadi A, Cheung KC. Investigation of the hydrodynamic response of cells in drop on demand piezoelectric inkjet nozzles. *Biofabrication* 2016;8:015008.
105. Christensen K, Xu C, Chai W, Zhang Z, Fu J, Huang Y. Freeform inkjet printing of cellular structures with bifurcations. *Biotechnology and Bioengineering* 2015;112:1047–1055.
106. Chang CC, Boland ED, Williams SK, Hoying JB. Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 2011;98:160–170.
107. Matsusaki M, Sakaue K, Kadowaki K, Akashi M. Three-dimensional human tissue chips fabricated by rapid and automatic inkjet cell printing. *Advanced Healthcare Materials* 2013;2:534–539.
108. Lee JW, Choi Y-J, Yong W-J, Pati F, Shim J-H, Kang KS et al. Development of a 3D cell printed construct considering angiogenesis for liver tissue engineering. *Biofabrication* 2016;8:015007.
109. Lee J-S, Hong JM, Jung JW, Shim J-H, Oh J-H, Cho D-W. 3D printing of composite tissue with complex shape applied to ear regeneration. *Biofabrication* 2014;6:024103.
110. Jang J, Kim TG, Kim BS, Kim S-W, Kwon S-M, Cho D-W. Tailoring mechanical properties of decellularized extracellular matrix bioink by vitamin B2-induced photocrosslinking. *Acta Biomaterialia* 2016;33:88–95.
111. Jang J, Park H-J, Kim S-W, Kim H, Park JY, Na SJ et al. 3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair. *Biomaterials* 2017;112:264–274.
112. Jung JW, Lee J-S, Cho D-W. Computer-aided multiple-head 3D printing system for printing of heterogeneous organ/tissue constructs. *Scientific Reports* 2016;6:21685.
113. Chang R, Nam J, Sun W. Direct cell writing of 3D microorgan for in vitro pharmacokinetic model. *Tissue Engineering Part C: Methods* 2008;14:157–166.
114. Chang R, Emami K, Wu H, Sun W. Biofabrication of a three-dimensional liver microorgan as an in vitro drug metabolism model. *Biofabrication* 2010;2:045004.
115. Koch L, Deiwick A, Schlie S, Michael S, Gruene M, Coger V et al. Skin tissue generation by laser cell printing. *Biotechnology and Bioengineering* 2012;109:1855–1863.
116. Pagès E, Rémy M, Kériquel V, Correa MM, Guillotin B, Guillemot F. Creation of highly defined mesenchymal stem cell patterns in three dimensions by laser-assisted bioprinting. *Journal of Nanotechnology in Engineering and Medicine* 2015;6:021006.



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Chapter 14 Bioprinting-enabled technologies for cryopreservation

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14.1 Introduction

Cryopreservation is a storage method for preserving the cells, aggregates, tissues, and organs for an extended period of time in a frozen state. The biological samples are kept at a low temperature while still maintaining their genetic characteristics and functional properties, such as proliferation and differentiation. The cryopreservation process follows four steps, including: (1) cryoprotectant (CPA) loading, (2) freezing, (3) thawing, and (4) CPA unloading (Shi et al. 2015, Tsai and Lin 2012, Medeiros et al. 2002, Day and Stacey 2007, Zhang et al. 2011, Yong et al. 2015, Demirci and Montesano 2007). The importance and effectiveness of

cryopreservation depends on its application, as well as the type of the cells and tissues, which are going to be preserved. The main role of this technique is to prevent the need to have all cell lines in culture at all times by storing stocks of the cells, especially for such cells, which have a limited life time. Cryopreservation aims to diminish consumable costs, the risk of microbial and cross contamination, as well as the risk of genetic drift and morphological changes.

Oocyte, sperm, stem cells, and their derivatives are examples of critical mammalian cells used in cell-based medical disciplines, such as tissue engineering (TE), regenerative medicine, and assisted reproduction. Therefore, finding an effective method for long-term storage of these cells is a crucial issue (He et al. 2008). The importance of the cryopreservation for the mentioned mammalian cells is shortly summarized in the following sections.

14.1.1 Sperm

Sperm cryopreservation has the potential to accelerate the rate of genetic selection and improve production by the expansion of reproductive techniques, such as artificial insemination (AI) and *in vitro* fertilization (IVF). It can provide the accessibility to sperm regardless of time and location (Medeiros et al. 2002, Tasoglu et al. 2013b, Knowlton et al. 2015a).

14.1.2 Oocyte

Cryopreservation of oocytes, as an essential tool for fertility treatment (Kuleshova and Lopata 2002, Luo et al. 2015), can facilitate oocyte banking, which affects female fertility. It can also be a replacement for embryo cryopreservation, which is especially important for cancer patients who have lost their ovarian function during their cancer treatment, patients with objections to embryo preservation, and women who do not currently have a partner but wish to bank oocytes for later use (Zhang et al. 2012, Choi et al. 2015a).

14.1.3 Embryos and embryonic cells

Successful cryopreservation of embryos provides several advantages for patients, above and beyond improving cumulative pregnancy rates. First, since the procedure of transferring the embryos is costly and difficult for the patient, it is considerably valuable to be able to limit the number of transferred embryos and maximize the usable embryo-per-oocyte-retrieval-cycle ratio at each stimulation attempt. This can be accomplished by the cryopreservation of embryos. Second, less drug stimulation cycles can be used to obtain oocytes, thereby decreasing the related subsequent risks. In addition, by retrieving and storing the eggs when the patients are younger, they are able to achieve a pregnancy at an advanced maternal age (Kuwayama et al. 2005a). Cryopreservation of gametes, embryos, and

embryonic cells in aquaculture and aquatic biotechnologies also plays a vital role, which can have an impact on seed production, conservation of aquatic resources, and protection of endangered species and genetic diversity in aquatic species (Tsai and Lin 2012).

14.1.4 Stem cells

Stem cells can be achieved in large numbers by expanding the cells in culture or by pooling them from a different donor. Since they are ideal for many biomedical applications, such as cell-based therapies and regenerative medicine, development and widespread use, as well as off-the-shelf availability, of these cells are important factors, which may be provided by using an appropriate long-term preservation method. Furthermore, to establish a cell bank, an appropriate cryopreservation method is essential. To have a reliable method to cryopreserve stem cells, it is necessary to minimize the risk of cryoinjury and to optimize the concentration and type of CPA (Yong et al. 2015, Reubinoff et al. 2001).

Since cryoinjury occurs at a low temperature due to ice formation, a cryoprotectant agent is used to protect the cells. In fact, CPAs play the role of osmotic buffers for preventing harmful critical electrolyte concentration gradients. Furthermore, they help the cell membranes stabilize and prevent macromolecules from changing from their native form. However, using a high concentration of CPA may be toxic, so finding the optimal concentration is crucial. CPAs should be as highly permeable and nontoxic as possible. Adding sugars (e.g., sucrose and trehalose), polymers (e.g., polyvinylpyrrolidone and polyvinyl alcohol), or other nonpermeable CPAs are possible options that decrease the toxicity of CPAs. Another way is to perform a stepwise loading of CPAs with lower concentrations to minimize the toxicity and osmotic shock. Dimethylsulphoxide (DMSO), 1,2-propanediol (PROH), ethylene glycol (EG), sucrose, trehalose, and mannitol are a number of the widely used CPAs (Dou et al. 2015, Zhang et al. 2011, Sheikhi et al. 2013, Selman 2005). CPAs used in cryopreservation studies coupled with the bioprinting method are presented in [Table 14.1](#).

There are four steps involved in cryopreservation, including CPA loading, freezing, thawing, and CPA unloading, which are affected by three factors: cooling rate, viscosity (CPA concentration), and sample size. By increasing the cooling rate and viscosity, as well as decreasing sample size, the vitrification probability will increase. Cryopreservation can be done in two ways: (1) slow freezing and (2) vitrification. In slow freezing, the cells are cooled down to a temperature below the freezing point with a controlled rate and low concentration of CPA to prevent ice formation and damage to the cell membrane (Zhang et al. 2011, Day and Stacey 2007, Blackburn et al. 2012, Barbas and Mascarenhas 2009, Demirci and Montesano 2007, Pyne et al. 2014, Dou et al. 2015). The vitrification method has attracted great attention due to its considerable advantages and

Table 14.1 CPAs Used in Cryopreservation Coupled with Bioprinting Method

Name	Commercially Available (Yes/No)	Concentration	Cell Type Used	Suspending Media	References
Glycerol	Y	2.5 M	Red Blood Cells (RBCs)	DI	(Samot et al. 2011)
Cryoink ^a	N	4.5 vol%	RBC	—	(El Assal et al. 2014)
PROH	Y	1.5 M	AML 12, HL1, NIH-3T3, mES, RAJI	BSA-supplemented PBS	(Demirci and Montesano 2007)
DMSO	Y	10 vol%	Mouse embryonic fibroblast cells (NIH 3T3 cells) and hASCs	Dulbecco's Modified Eagle's Medium (DMEM)	(Shi et al. 2015)
DMSO	Y	2.5 M	Oocyte	FHM HEPES buffered medium supplemented with BSA	(Zhang et al. 2012)
DMSO/PEG	Y	5.5–10 vol%	3T3 fibroblast cell line and Neuroprogenitor cells (NPCs) were derived from hESC line HUES7	Culture Media	(Dou et al. 2015)

^a This cryoink contains ectoine (9% v/v), trehalose (25 µg mL⁻¹), and polyethylene glycol (PEG, 1% v/v).

improved outcome, as compared to slow freezing method. A high cooling rate coupled with a higher concentration of CPA is needed to avoid intracellular ice creation and cytoskeleton disruption (Dou et al. 2015, Aquino et al. 2014). The cooling rate in the process determines the rate and extent of water movement and ice nucleation. Therefore, the fast cooling rate of the vitrification method leads to a lower degree of dehydration and is the most appropriate for cells, which are sensitive to high solute concentration (Amorim et al. 2011). The cooling rate and thawing rate should each be higher than the critical cooling rate and critical warming rate, respectively (Zhou et al. 2013). A high thawing rate is needed for the prevention of devitrification (turning to ice from a glass) (Jin et al. 2014).

14.2 Vitrification versus slow freezing method

By using the slow freezing method, the cells suffer from damages due to dehydration, intra- and extracellular ice crystal formation, and extreme hyperosmolarity. Intracellular ice crystallization can have a deleterious effect on the cell wall and structure (Mphaphathi et al. 2012, Selman 2005, Zhang et al. 2011, Yong et al. 2015). Furthermore, the slow freezing method is time-consuming and requires expensive equipment to control the freezing rate. In contrast, vitrification is a simple, easy, and fast process, which transforms the cell suspension from an aqueous phase to the high viscose amorphous phase of the vitrified state (Day and Stacey 2007, Barbas and Mascarenhas 2009, Demirci and Montesano 2007, Amorim et al. 2011, Yong et al. 2015, Pyne et al. 2014, Kuwayama et al. 2005a, Criado et al. 2011). Vitrification forms no ice crystals, thereby eliminating the concern of cell death caused by ice formation (Kuwayama et al. 2005a, Pyne et al. 2014, Amorim et al. 2011). Moreover, there is no need for special equipment, making it a cost-effective method in which the necessary skills can be learned in a short time. Occasionally, during the vitrification process, the operator needs to observe the cells to be able to recognize the viable cells. For example, nonviable zygotes and blastomeres do not contract in the vitrification solution, so they can be recognized and identified during the thawing process by comparison with viable cells (Shi et al. 2015, Selman 2005, Barbas and Mascarenhas 2009, Amorim et al. 2011, Pyne et al. 2014). Despite the great advantages of the vitrification technique, some challenges are also encountered. Some possible issues include the following: difficulty of reaching high cooling and thawing rates, risk of contamination due to the direct contact of the specimen with nonsterile liquid nitrogen, challenges of dealing with large-size specimens, and the need to use a higher CPA concentration, which can cause osmotic shock and toxicity (Tavukcuoglu et al. 2012, Samot et al. 2011, Shi et al. 2015, Ehrlich et al. 2015, Zhang et al. 2011). Vitrification falls into two approaches: (1) equilibrium and (2) nonequilibrium. In equilibrium vitrification, by the stepwise introduction of CPA, chemical toxicity will be lowered, though osmotic damage to the cells is still probable.

Nonequilibrium vitrification employs a high cooling rate coupled with lower concentrations of CPA. Vitrification systems in this approach are divided into two classes: (1) carrier-based systems and (2) carrier-free systems. The aim of using carriers such as straw (Kuwayama et al. 2005b, Chen et al. 2001, Hurtt et al. 2000, Isachenko et al. 2005), quartz microcapillary (Choi et al. 2015a, 2015b, He et al. 2008, Criado et al. 2011), cryoloop (Mukaida et al. 2001, Takahashi et al. 2005, Lane et al. 1999), cryotop (Valbuena et al. 2012, Kuwayama et al. 2005b, Cobo et al. 2008), cryotip (Kuwayama et al. 2005a, Valbuena et al. 2012, da Motta et al. 2014, Bianchi et al. 2014), cryoleaf (Panagopoulou et al. 2013, Wang et al. 2012, Combelles et al. 2011), and cryolock (García et al. 2011, Casillas et al. 2014, 2015) is to achieve a high cooling rate and to prevent cryoinjury. Figure 14.1 shows some of these carriers. Each carrier-based system has a different cooling rate. For example, plastic straws provide a cooling rate at $2500^{\circ}\text{C}/\text{min}$, quartz microcapillaries offer $250,000^{\circ}\text{C}/\text{min}$, and by cryoloops, a cooling rate as high as $700,000^{\circ}\text{C}/\text{min}$ can be achieved (Yong et al. 2015). Furthermore, microfluidics and its applicability to cryopreservation have been investigated. Microfluidics have been demonstrated for controlling the concentration of CPA in the loading/unloading steps (Song et al. 2009), automating the preparation of cells for vitrification (Pyne et al. 2014), and as a method for cryoprotectant-free cryopreservation (Figure 14.2) (Zou et al. 2013).

Besides the mentioned advantages of vitrification—including the prevention of ice formation, reduction of damages due to the high cooling rate, being simple and cost-effective, as well as not requiring a programmed freezer—it also offers some

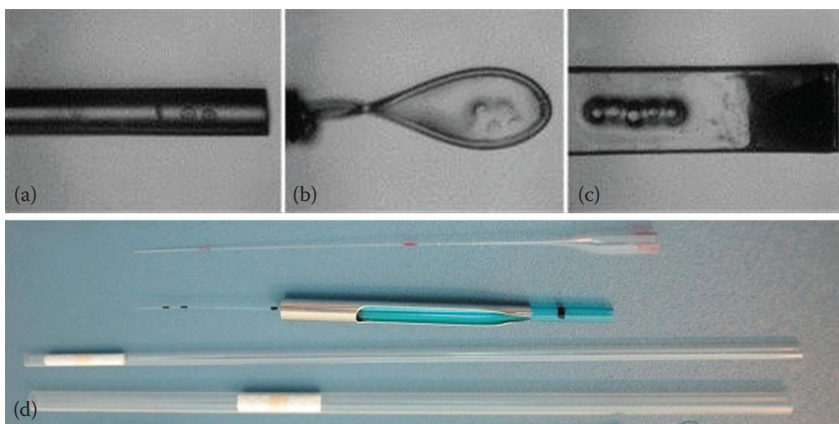


Figure 14.1 Illustration of the tip of cryodevices used in minimum volume vitrification methods. (a) Gel-loading tip. (b) Cryoloop. (c) Cryotop. (Reproduced from Zhang, X. et al., *Nanomedicine*, 6, 1115–1129, 2011. With permission.) (d) (top to bottom): quartz capillary, CryoTip, 0.25-mL straw, and 0.5-mL straw. (Reproduced from Criado, E. et al., *Fertil. Steril.*, 95, 1101–1103, 2011. With permission.)

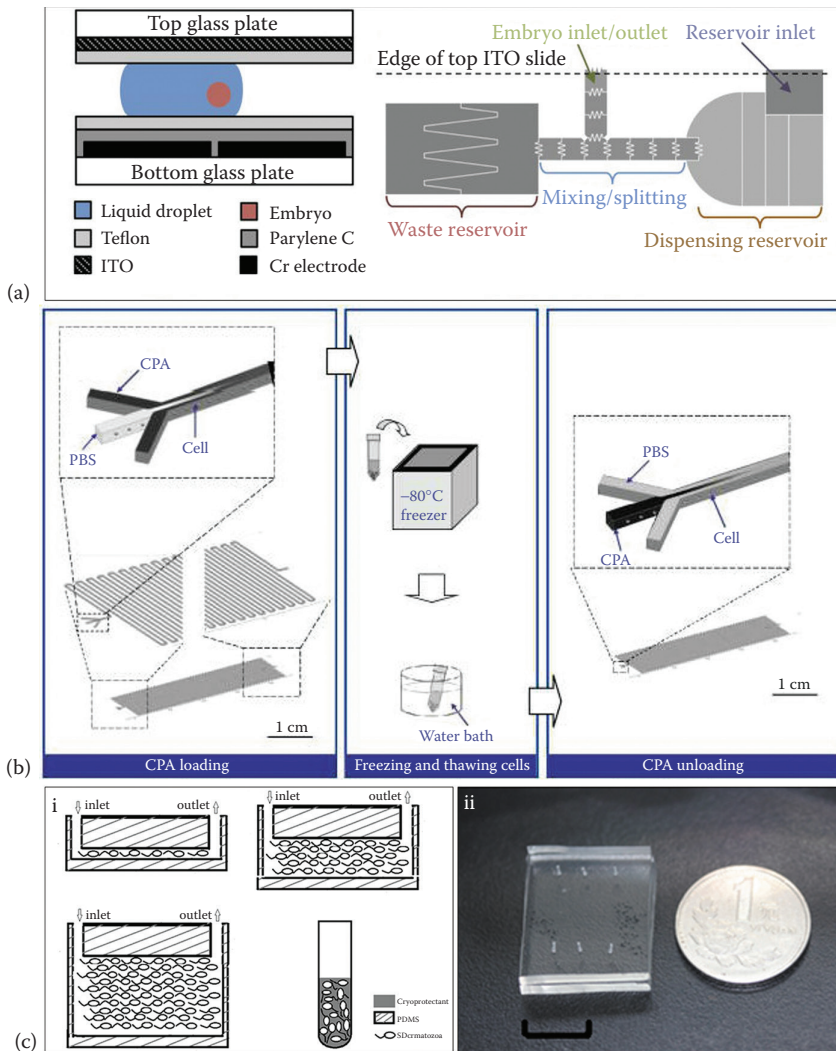


Figure 14.2 Schematic of used microfluidics in cryopreservation studies. (a) Key device design elements and fabrication layers of digital microfluidic system used in vitrification. (Reproduced from Pyne, D.G. et al., *PLoS One*, 9, e108128, 2014. With permission.) (b) Schematic depiction of cell cryopreservation with the microfluidic approach consists of three steps, CPA loading, freezing and thawing, and CPA unloading. (Reproduced from Song, Y.S. et al., *Lab on a Chip*, 9, 1874–1881, 2009. With permission.) (c) (i) Schema of spermatozoa store in PDMS chip with different height and in a freezing tube; (ii) The PDMS chip used in the study. (Reproduced from Zou, Y. et al., *PLoS One*, 8, e61593, 2013. With permission.)

Table 14.2 Characteristics of Vitrification versus Slow Freezing Method

Characteristics	Slow Freezing	Vitrification
Concentration of CPA and risk of toxicity with CPAs	Low	High
Potential contamination with pathogenic agents	Low	High
Special equipment	Yes	No
Ice formation and risk of freeze injury	Yes	No
Required labor skill	Low	Medium

Source: Yong, K.W. et al., *Biopreserv. Biobank.*, 13, 231–239, 2015; Amorim, C.A. et al., *Reprod. Biomed.Online*, 23, 160–186, 2011.

advantages over the slow freezing method regarding the viability, genetic profiles, and cytoskeletal structure of cells. This superiority of the vitrification method has made it a great technique, which leads to better cryopreservation results in various applications in biomedical fields such as reproduction, TE, and organ transplantation (Shi et al. 2015, Tavukcuoglu et al. 2012, Mphaphathi et al. 2012, Sheikhi et al. 2013). [Table 14.2](#) briefly compares vitrification with slow freezing method.

14.3 Theoretical analysis

14.3.1 Heat transfer

Among the variables that have an effect on the vitrification outcome are the rate of cooling and thawing, the sample size, and the type and concentration of CPA (Amorim et al. 2011). Decreasing the volume of the droplet allows for a higher cooling rate by reducing the heat resistance. Heat resistance of a cell-laden droplet can be calculated from the following equation:

$$Heat\ resistance = \frac{(r_{droplet} - r_{cell})}{4\pi k r_{droplet} \times r_{cell}} \tag{14.1}$$

where:

- $r_{droplet}$ is the droplet radius
- r_{cell} is the radius of the cell residing in the droplet
- k ($Wm^{-1}K^{-1}$) is the thermal conductivity

A large droplet has a large heat resistance, which leads to a low cooling rate and therefore needs a higher CPA concentration (Demirci and Montesano 2007). Using pico- or nanoliter droplets also decrease the effect of Leidenfrost’s phenomenon in open systems, which have direct contact with liquid nitrogen. On the other hand, the high cooling rate reduces the need for a high CPA concentration, consequently decreasing the risk of toxicity. Therefore, reducing droplet volume can potentially decrease the risk of toxicity, increase the overall success of the procedure, and improve the efficiency of cryopreservation (Shi et al. 2015, Zhang et al. 2011, 2012, Amorim et al. 2011, Dou et al. 2015, Scholz 2012).

Vitrification can be considered as two coupled processes: (1) heat transfer and (2) crystallization. Heat transfer during the cryopreservation method is frequently expressed by the following equation, which is based on Fourier's law:

$$\rho c_p \frac{\partial T}{\partial t} = k \nabla^2 T + \dot{\phi} + \dot{q}_{ext} \quad (14.2)$$

where:

T (K) and t (s) are temperature and time, respectively

ρ (kg m^{-3}) and c_p ($\text{J kg}^{-1}\text{K}^{-1}$) are the density and specific heat, respectively

$\dot{q}_{ext}$ (W m^{-3}) is the external heat source and $\dot{\phi}$ (W m^{-3}) is the metabolic heat generation

Equation 14.2 is coupled with the following equation (14.3):

$$\frac{\partial T}{\partial t} = \alpha \nabla^2 T + \frac{L}{c_p} \frac{d\chi}{dt} \quad (14.3)$$

where:

L is the latent heat

χ is degree of crystallization (Xu et al. 2010, Shi et al. 2015)

Fourier's law is based on the assumption of infinite speed of propagation of thermal waves, which is not physically achievable. Therefore, it demonstrates a failure in the modeling of the processes: materials with nonhomogeneous inner structure suffer from the propagation speed of the thermal waves, which is not high enough. To address this limitation, the thermal wave model with hyperbolic heat equation was introduced while considering the finite propagation speed.

$$q(r, t + \tau_q) = -k \nabla T(r, t) \quad (14.4)$$

where q (W m^{-2}) is the heat flux vector representing heat flow per unit time, per unit area of the isothermal surface in the direction of the decreasing temperature and r stands for the position vector. τ_q is the thermal relaxation time that can be interpreted as the phase lag between heat flux and temperature gradient in high rate response:

$$\tau_q = \alpha / C_t^2 (s) \quad (14.5)$$

where α ($\text{m}^2 \text{s}^{-1}$) and C_t (m s^{-1}) are the thermal diffusivity and the speed of thermal wave in the medium, respectively.

Although this model has considered the finite propagation speed, its validity has been questioned. One of the limitations of the thermal wave model is that it ignores the effect of microstructural interactions on the fast transient process of heat transport (e.g., fast cooling during cryopreservation processes). Therefore,

the dual phase-lag model was presented as follows to overcome this limitation of the thermal wave model:

$$q(r, t + \tau_q) = -k \nabla T(r, t + \tau_T) \quad (14.6)$$

where τ_T and τ_q can be considered as the periods arising from *microstructural interaction* and *thermal inertia*, respectively (Xu et al. 2010).

14.3.2 Crystallization

Now, it is well accepted that the lower the degree of crystallization leads to the more efficient vitrification. The following nonisothermal kinetic equation is used to describe the crystallization process:

$$\frac{d\chi}{dt} = k_a \chi^{\frac{2}{3}} (1 - \chi) (T_m - T) e^{-Q/RT} \quad (14.7)$$

where:

χ is the degree of crystallization (which changes from zero to one)

t (s) is the time

T and T_m (K) are temperature of droplet and final temperature of the freezing process

Q (J mol⁻¹) and R (J mol⁻¹K⁻¹) are the activation energy and gas constant, respectively

k_a represents the characteristic coefficient of the crystallization and is expressed by

$$k_a = \frac{L}{\pi} \delta^2 \upsilon T_m r_f$$

where:

L (J kg⁻¹) is the latent heat

δ (m) is the thickness of the transition layer between liquid and solid

r_f (m) is the radius of the ice region

υ (m² s⁻¹) is the kinematic viscosity (Xu et al. 2010, Shi et al. 2015)

14.4 Droplet-based bioprinting technologies

Bioprinting technologies hold great promise for application to cryopreservation. It is a method of generating patterns of living cells and biological materials onto a receiving substrate, with spatial control, using printing technologies (Knowlton et al. 2017, Lepowsky and Tasoglu 2018). It is a method of generating patterns of living cells and biological materials onto a receiving substrate, with spatial control, using printing technologies. Successive layer-by-layer precise patterning offers a unique ability to fabricate tissue-like three-dimensional (3D) assemblies composed of living cells for regenerative medicine (Tasoglu et al. 2013a, 2014a, 2014b, Gurkan et al. 2012, Knowlton et al. 2015b, Durmus et al. 2013, Chen et al. 2014,

Knowlton et al. 2016d, Knowlton et al. 2016b, Knowlton et al. 2016a, Knowlton et al. 2016c, Knowlton et al. 2017, Tasoglu and Demirci 2013, Guven et al. 2015). Within the field of bioprinting, the main methods used for the deposition and patterning of living cells are inkjet bioprinting (Boland et al. 2006, Nakamura et al. 2005), microextrusion (Fedorovich et al. 2009, Chang et al. 2008, 2011, Khalil and Sun 2007), and laser-assisted bioprinting (Odde and Renn 1999, Nahmias et al. 2005, Guillotin et al. 2010, Gaebel et al. 2011, Barron et al. 2004). Droplet-based (digital) bioprinting methods have been widely used for cryopreservation with the goal of increasing rate of cooling. Therefore, we review droplet-based bioprinting methods in this section based on the principles of inkjet and laser printing technologies (Figure 14.3), and their important features such as cell viability, resolution, and material viscosity (Table 14.3).

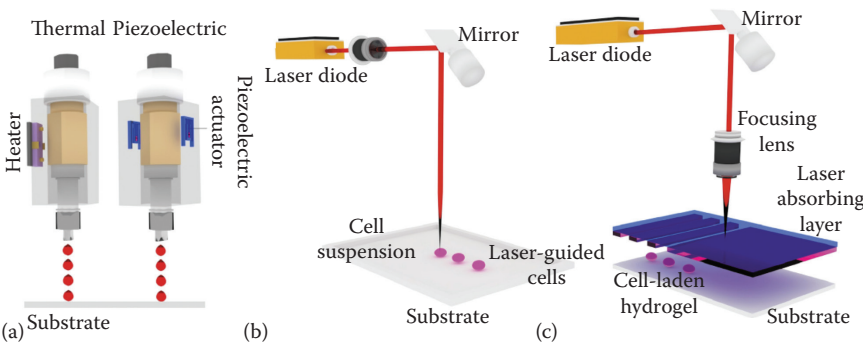


Figure 14.3 Illustration of droplet-based bioprinting methods based on the principles of inkjet and laser technologies. (a) Thermal and piezoelectric inkjet printing. (b) Laser-guided direct cell printing. (c) Laser-induced direct cell printing. (Reproduced from Knowlton, S. et al., *Trends Biotechnol.*, 33, 504–513, 2015b. With permission.)

Table 14.3 Characteristics of Inkjet versus Laser-Assisted Bioprinting

Performance Metric	Inkjet Bioprinting	Laser-Assisted Bioprinting
Cell viability (%)	60–85 (Xu et al. 2005, Xu et al. 2006, Chang et al. 2008)	80–85 (Hopp et al. 2005)
Cell density (cells/ml)	$<5 \times 10^6$ (Guillotin and Guillemot 2011, Xu et al. 2005)	6×10^7 – 1×10^8 (Guillotin et al. 2010, Guillotin and Guillemot 2011)
Resolution (μm)	~75 (Campbell et al. 2005, Phillippi et al. 2008)	~50 (Guillotin et al. 2010)
Material viscosity (mPa.s)	<10 (Kim et al. 2010)	1–300 (Guillotin and Guillemot 2011)
Print speed	Medium (Malda et al. 2013)	Low (Malda et al. 2013)
Printer cost	Low (Jones 2012)	High (Jones 2012)
Throughput	High (Tasoglu and Demirci 2013)	Medium (Tasoglu and Demirci 2013)

Inkjet bioprinting involves the dispensing and precise positioning of droplets of bioink through a nozzle onto a substrate (Murphy and Atala 2014). The two common approaches of inkjet bioprinting are thermal printing and piezoelectric actuated printing, both of which expel droplets from the nozzle (Nakamura et al. 2005, Xu et al. 2006). Thermal inkjet printers generate pulses of pressure by vaporizing a small volume of the bioink to generate droplets. However, mammalian cells are sensitive to heat, and the localized heat of the nozzle (200°C–300°C) has been shown to have a minor adverse influence on the stability and viability of cells (Boland et al. 2006). Piezoelectric inkjet printers generate mechanical pulses in the form of an acoustic wave that forces the bioink to form droplets, which is a more biocompatible droplet forming method (Nakamura et al. 2005). In inkjet printing technology, the bioink viscosity has an upper bound, which is limited to prevent the nozzle from clogging (Kim et al. 2010, Xu et al. 2005). To develop a better understanding of inkjet printing technology and resulting cell viabilities, fluid dynamic models have been presented (Muradoglu and Tasoglu 2010, Tasoglu et al. 2010). Results demonstrated that Weber number and geometric deformation of cells are highly correlated.

The laser-induced forward transfer (LIFT) technique is the most common laser-assisted bioprinting method. In this approach, a donor substrate, which is deposited with a laser energy absorbing layer and a film of cell-encapsulated bioink, is placed parallel to collector substrate. The laser pulses result in the local evaporation of the absorbing layer that, in turn, creates a high-pressure vapor, which transfers the bioink droplet onto the collector substrate (Guillotin et al. 2010, Barron et al. 2004, Colina et al. 2006, Kattamis et al. 2009). The laser-guided direct cell printing method, another laser-assisted bioprinting approach, uses a laser beam to trap and direct cells and then deposit them onto a nonabsorbing substrate (Odde and Renn 1999, Nahmias and Odde 2006, Guillemot et al. 2010). These laser-assisted approaches are nozzle-free techniques and are not restricted by clogging issues; therefore, high viscosity biomaterials could be used as bioink (Guillotin and Guillemot 2011). Previous studies have shown that laser-assisted bioprinting does not affect the viability, survivability, or DNA function of cells (Hopp et al. 2005, Guillotin et al. 2010).

Bioprinting technologies have enabled the high-throughput manipulation of cells via nanoliter droplets that empowered a new cryopreservation approach called cryoprinting (Tasoglu and Demirci 2013). Using inkjet bioprinting for cell delivery and handling, followed by rapid freezing/vitrification, cryoprinting results in the processing of large cell numbers with good viability and survivability (Dou et al. 2015, Wang et al. 2013). The valve-based droplet-ejection method is another droplet-based bioprinting approach that is highly used in cryoprinting (Demirci and Montesano 2007, Song et al. 2010). In this method, a pulsatile opening valve ejects pressurized bioinks in the form of droplets onto a collector substrate, located in liquid nitrogen bath.

14.5 Droplet-based vitrification

Recently, droplet-based vitrification as a valuable cryopreservation method has led to successful results in preserving biological specimens. Due to the small volume, in addition to providing high cooling and rewarming rates, which prevent ice crystal formation, there is no need to use a high concentration of CPAs. Therefore, using bioprinters to transform bulk samples to uniform pico- or nanoliter droplets can address some of the limitations of the vitrification method. Some of the research involving droplet-based vitrification is summarized in the following sections and is illustrated in [Figure 14.4](#).

14.5.1 Anti-Leidenfrost vitrification

Encapsulating single cells while preserving the highest viability possible (>90%) has a great impact on different research fields, especially TE, high-throughput screening, as well as clinical diagnostics and therapeutics. Demirci and Montesano (2007) showed successful vitrification of a small number of cells using cell encapsulation. With this novel method, they managed to vitrify the cells with a high cooling rate and a CPA concentration as low as that used in slow freezing protocols. They showed that this method significantly decreased osmotic and mechanical stresses on cells. To overcome the limitations of the droplet vitrification method, Zhang et al. (2012) also introduced an ejector-based droplet vitrification system that continuously generated nanoliter droplets. This scalable vitrification was used for mouse oocyte cryopreservation, which enabled an ultrarapid cooling rate with a reduction in the required concentration of CPA by using nanoliter droplets. The researchers confirmed that droplet encapsulation did not significantly affect the oocyte survival or parthenogenetic embryo development, in addition to demonstrating the efficiency of the presented approach.

Cryopreservation of red blood cells (RBC) as a method for providing blood banking has a great effect on overcoming blood shortages, which frequently occur during natural disasters and military conflicts. Although vitrification has potential advantages, it has not yet achieved a broad application because of some challenges. To overcome some of the limitations in the cryopreservation of RBCs, Samot et al. (2011) introduced a high-throughput, ultrarapid method to vitrify RBCs in microdroplets, which collectively had the ability of vitrifying blood in large volume but a short time. To fabricate microdroplets for encapsulating the cells, the researchers used an ejector-based printer and investigated the effect of three parameters on the droplet size, including droplet collection distance (from ejector tip to droplet collection film), flow rate of nitrogen gas, and flow rate of CPA-loaded RBCs. The cryopreservation process included blood preparation, CPA loading, ejection, freezing, thawing, and collection.

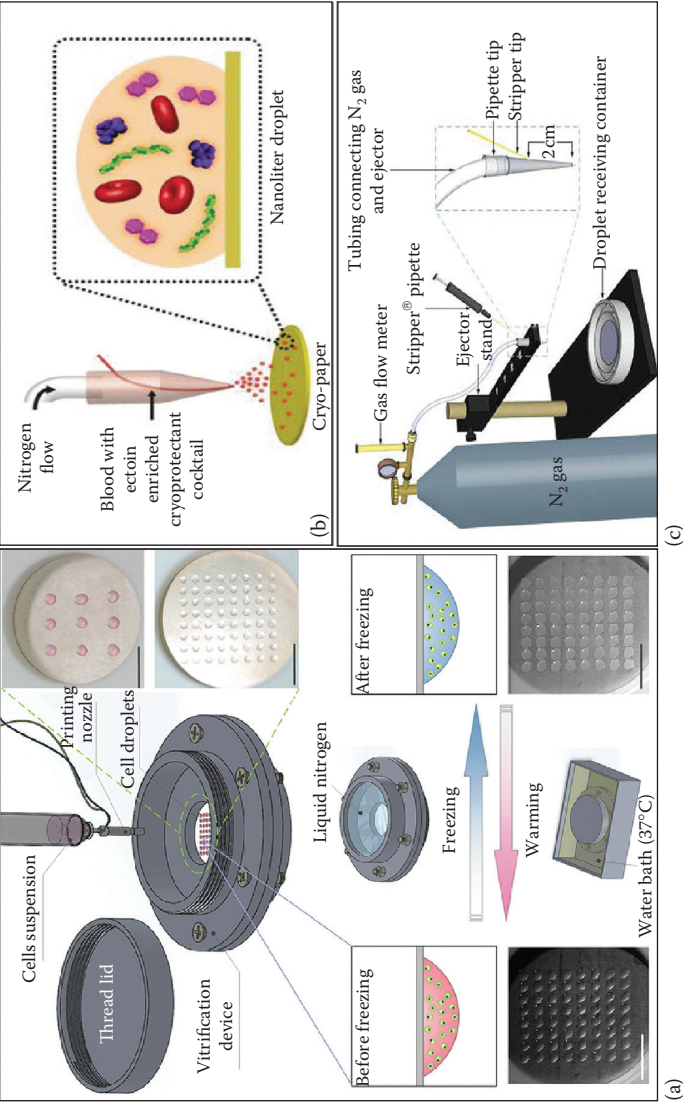


Figure 14.4 Illustration of the present bioprinter technologies used for cryopreservation studies. (a) Vittrification of cell-laden droplets based on cell printing. (scale bars: 10 mm). (Reproduced from Shi, M. et al., *Sci. Rep.*, 5, 2015. With permission.) (b) Droplet-based vitrification of red blood cells by using ejector-based bioprinter. (Reproduced El Assal, R. et al., *Adv. Mater.*, 26, 5815–5822, 2014. With permission.) (c) The ejector-based droplet generation setup. (Reproduced Zhang, X. et al., *Nanomedicine*, 7, 553–564, 2012. With permission.) (Continued)

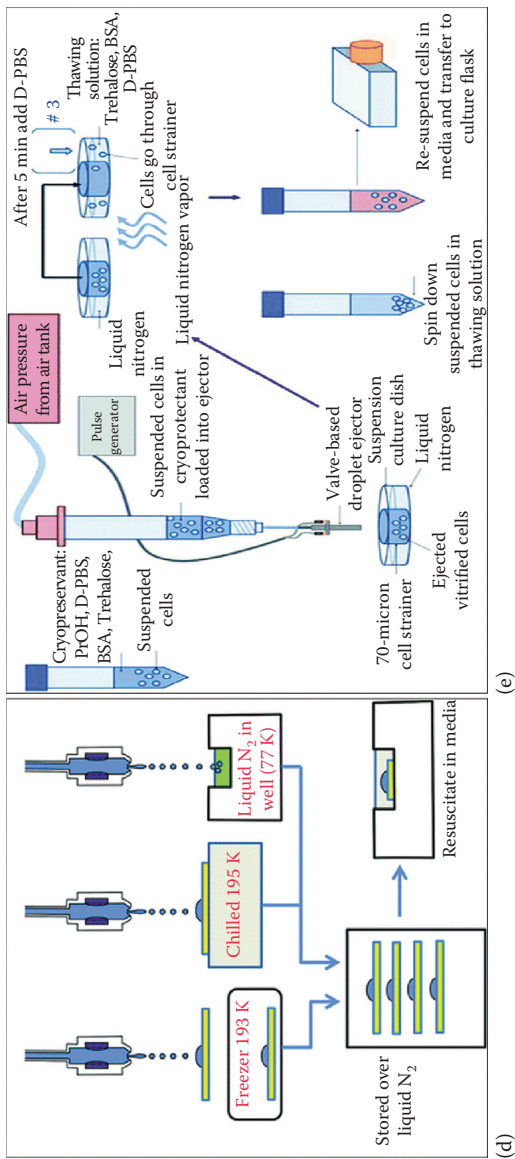


Figure 14.4 (Continued) Illustration of the present bioprinter technologies used for cryopreservation studies. (d) Schematic of the three cryoprinting procedures. (Reproduced from Dou, R. et al., *Lab on a Chip*, 15, 3503–3513, 2015. With permission.) (e) Set up for droplet encapsulated cell vitrification. (Reproduced Demirci, U. and Montesano, G., *Lab on a Chip* 7, 1428–1433, 2007. With permission.)

Furthermore, in an effort to avoid the cryoinjury of RBCs occurring while using conventional vitrification, Assal et al. suggested an innovative vitrification approach using the bioprinting method. They used a cryoprinter for generating nanoliter droplets in which the cryoink acted as a cryoprotectant agent. Their results showed the potential of this method for biopreserving different cell types, as well as for improving the efficiency of blood banking. They also proposed using multiple ejectors for rapid printing of droplets for bulk volumes of blood samples, in addition to utilizing sterilized liquid nitrogen to minimize the contamination risks of the cells as a challenge of vitrification (El Assal et al. 2014).

Dou et al. (2015) also used inkjet printing and successfully cryopreserved 3T3 mouse fibroblast cells and human neuroprogenitor cells (NPCs) via the formation of nanodroplets. They demonstrated cryopreservation by inkjet printing as a high-throughput method, which needed lower concentrations of CPA. DMSO was used as a CPA with a concentration less than 0.8 M and the viability of the cells was observed for 6 months. They tested three different methods for the cooling process, including printing the cells on a polymer substrate followed by the transfer to a freezer held at 293 K, printing the cells on a polymer substrate on a metal base held at 195 K, and printing directly into the liquid nitrogen. They used a mixture of DMSO and polyethylene glycol (PEG) as a CPA at a low deposition rate to keep the viability of the cells high enough after freezing.

14.5.2 Contactless vitrification

Direct contact of cells with liquid nitrogen is one of the concerns regarding the vitrification process because it has the potential of contamination and poses a difficulty for subsequent cell collection. To address this concern, a novel noncontact vitrification method was introduced utilizing an ultrathin silver film with high thermal conductivity. By this method, a high cooling rate was achieved without direct contact of cells and liquid nitrogen, while also mitigating the cell collection problem. Shi et al. (2015) also showed the benefit of small volume droplets for increasing the cooling and warming rates. They used a bioprinter to generate cell-laden microdroplets on one side of the silver film and poured liquid nitrogen on the other side. It was shown that using this device was an effective way for high-throughput cryopreservation while still addressing the aforementioned contamination and collection issues.

14.6 Conclusions and future perspective

Vitrification is a promising method for preserving biological specimens. To address some of the limitations of this method, droplet-based vitrification is introduced. By decreasing the volume of the sample, the cooling rate increases, thereby

decreasing the need for high CPA concentrations. Applying low-concentration CPA in droplet-based vitrification will significantly decrease cell damage caused by osmotic shock and chemical toxicity, which are the main concerns in traditional vitrification. By comparing the results of viability after the thawing step, it is demonstrated that the droplet-based vitrification method leads to the higher cell viability.

Since having droplets with consistent size is crucial for achieving reliable and repeatable results, and the fact that the size of the droplet can affect the result in clinical testing, finding a method to satisfy these is indispensable. The results show that using bioprinters can lead to an automated way for generating droplets with consistency in size, and, most important, consistent cryopreservation. Moreover, using bioprinters with the capability of being modified enables the continuous encapsulation of cells at high rates, the generation of droplets, and the decreased role of the operator. The scalability potential of cell printers for generating bulk volume of droplets can eventually lead to the use of such devices in clinical settings. These improvements make the vitrification process efficient and cost-effective.

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References

- Amorim, C. A., M. Curaba, A. Van Langendonck, M. M. Dolmans, and J. Donnez. 2011. Vitrification as an alternative means of cryopreserving ovarian tissue. *Reproductive Biomedicine Online* 23 (2):160–186.
- Aquino, D., L. Danielli, P. Rigon, N. Lothhammer, N. Frantz, and A. Bos-Mikichi. 2014. Ovarian tissue vitrification: The use of a novel metal closed system for clinical grade cryopreservation. *JBRA Assisted Reproduction* 18:12–15.
- Barbas, J. P., and R. D. Mascarenhas. 2009. Cryopreservation of domestic animal sperm cells. *Cell and Tissue Banking* 10 (1):49–62.
- Barron, J. A., B. R. Ringeisen, H. Kim, B. J. Spargo, and D. B. Chrisey. 2004. Application of laser printing to mammalian cells. *Thin Solid Films* 453:383–387.

- Bianchi, V., G. Macchiarelli, A. Borini, M. Lappi, S. Cecconi, S. Miglietta, G. Familiari, and S. A. Nottola. 2014. Fine morphological assessment of quality of human mature oocytes after slow freezing or vitrification with a closed device: A comparative analysis. *Reproductive Biology and Endocrinology* 12:110.
- Blackburn, H., R. Godke, P. Purdy, S. J. Hiemstra, H. Woelders, A. Mariante, F. Pizzi, and P. Boettcher. 2012. Cryoconservation of animal genetic resources. *Complete Book* (12):222.
- Boland, T., T. Xu, B. Damon, and X. Cui. 2006. Application of inkjet printing to tissue engineering. *Biotechnology Journal* 1 (9):910–917. doi: 10.1002/biot.200600081.
- Campbell, P. G., E. D. Miller, G. W. Fisher, L. M. Walker, and L. E. Weiss. 2005. Engineered spatial patterns of FGF-2 immobilized on fibrin direct cell organization. *Biomaterials* 26 (33):6762–6770.
- Casillas, F., Y. Ducolomb, A. E. Lemus, C. Cuello, and M. Betancourt. 2015. Porcine embryo production following in vitro fertilization and intracytoplasmic sperm injection from vitrified immature oocytes matured with a granulosa cell co-culture system. *Cryobiology* 71 (2):299–305.
- Casillas, F., M. Teteltitla-Silvestre, Y. Ducolomb, A. E. Lemus, Z. Salazar, E. Casas, and M. Betancourt. 2014. Co-culture with granulosa cells improve the in vitro maturation ability of porcine immature oocytes vitrified with cryolock. *Cryobiology* 69 (2):299–304.
- Chang, C. C., E. D. Boland, S. K. Williams, and J. B. Hoying. 2011. Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 98 (1):160–170. doi:10.1002/jbm.b.31831.
- Chang, R., J. Nam, and W. Sun. 2008. Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. *Tissue Engineering Part A* 14 (1):41–48. doi:10.1089/ten.a.2007.0004.
- Chen, P., Z. Luo, S. Güven, S. Tasoglu, A. V. Ganesan, A. Weng, and U. Demirci. 2014. Micro-scale assembly directed by liquid-based template. *Advanced Materials* 26 (34):5936–5941.
- Chen, S. U., Y. R. Lien, Y. Y. Cheng, H. F. Chen, H. N. Ho, and Y. S. Yang. 2001. Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. *Human Reproduction* 16 (11):2350–2356.
- Choi, J. K., H. Huang, and X. He. 2015a. Improved low-CPA vitrification of mouse oocytes using quartz microcapillary. *Cryobiology* 70 (3):269–272.
- Choi, J. K., T. Yue, H. Huang, G. Zhao, M. Zhang, and X. He. 2015b. The crucial role of zona pellucida in cryopreservation of oocytes by vitrification. *Cryobiology* 71 (2):350–355.
- Cobo, A., M. Kuwayama, S. Pérez, A. Ruiz, A. Pellicer, and J. Remohí. 2008. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertility and Sterility* 89 (6):1657–1664.
- Colina, M., M. Duocastella, J. M. Fernandez-Pradas, P. Serra, and J. L. Morenza. 2006. Laser-induced forward transfer of liquids: Study of the droplet ejection process. *Journal of Applied Physics* 99 (8):7. doi: 10.1063/1.2191569.
- Combelles, C. M. H., S. T. Ceyhan, H. Wang, and C. Racowsky. 2011. Maturation outcomes are improved following Cryoleaf vitrification of immature human oocytes when compared to choline-based slow-freezing. *Journal of Assisted Reproduction and Genetics* 28 (12):1183–1192.
- Criado, E., E. Albani, P. V. Novara, A. Smeraldi, A. Cesana, V. Parini, and P. E. Levi-Setti. 2011. Human oocyte ultravitrification with a low concentration of cryoprotectants by ultrafast cooling: A new protocol. *Fertility and Sterility* 95 (3):1101–1103.

- da Motta, E. L., M. Bonavita, J. R. Alegretti, M. Chehin, and P. Serafini. 2014. Live birth after 6 years of oocyte vitrification in a survivor with breast cancer. *Journal of Assisted Reproduction and Genetics* 31 (10):1397–1400.
- Day, J. G., and G. Stacey. 2007. *Cryopreservation and Freeze-Drying Protocols*. Vol. 368: Springer Science & Business Media. Humana Press Inc., New Jersey, US.
- Demirci, U., and G. Montesano. 2007. Cell encapsulating droplet vitrification. *Lab on a Chip* 7 (11):1428–1433.
- Dou, R., R. E. Saunders, L. Mohamet, C. M. Ward, and B. Derby. 2015. High throughput cryo-preservation of cells by rapid freezing of sub- μ l drops using inkjet printing–cryoprinting. *Lab on a Chip* 15 (17):3503–3513.
- Durmus, N. G., S. Tasoglu, and U. Demirci. 2013. Bioprinting: Functional droplet networks. *Nature Materials* 12 (6):478–479.
- Ehrlich, L. E., J. S. G. Feig, S. N. Schiffres, J. A. Malen, and Y. Rabin. 2015. Large thermal conductivity differences between the crystalline and vitrified states of DMSO with applications to cryopreservation. *PloS One* 10 (5):e0125862.
- El Assal, R., S. Guven, U. A. Gurkan, I. Gozen, H. Shafiee, S. Dalbeyler, N. Abdalla, G. Thomas, W. Fuld, and B. M. W. Illigens. 2014. Bio-inspired cryo-ink preserves red blood cell phenotype and function during nanoliter vitrification. *Advanced Materials* 26 (33):5815–5822.
- Fedorovich, N. E., I. Swennen, J. Girones, L. Moroni, C. A. van Blitterswijk, E. Schacht, J. Alblas, and W. J. Dhert. 2009. Evaluation of photocrosslinked Lutrol hydrogel for tissue printing applications. *Biomacromolecules* 10 (7):1689–1696. doi: 10.1021/bm801463q.
- Gaebel, R., N. Ma, J. Liu, J. Guan, L. Koch, C. Klopsch, M. Gruene et al. 2011. Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. *Biomaterials* 32 (35):9218–9230. doi: 10.1016/j.biomaterials.2011.08.071.
- García, J. I., L. Noriega-Portella, and L. Noriega-Hoces. 2011. Efficacy of oocyte vitrification combined with blastocyst stage transfer in an egg donation program. *Human Reproduction* 26 (4):782–790.
- Guillemot, F., A. Souquet, S. Catros, B. Guillotin, J. Lopez, M. Faucon, B. Pippenger, R. Bareille, M. Rémy, and S. Bellance. 2010. High-throughput laser printing of cells and biomaterials for tissue engineering. *Acta Biomaterialia* 6 (7):2494–2500.
- Guillotin, B., and F. Guillemot. 2011. Cell patterning technologies for organotypic tissue fabrication. *Trends in Biotechnology* 29 (4):183–190.
- Guillotin, B., A. Souquet, S. Catros, M. Duocastella, B. Pippenger, S. Bellance, R. Bareille et al. 2010. Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials* 31 (28):7250–7256 doi: 10.1016/j.biomaterials.2010.05.055.
- Gurkan, U. A., S. Tasoglu, D. Kavaz, M. C. Demirel, and U. Demirci. 2012. Emerging technologies for assembly of microscale hydrogels. *Advanced Healthcare Materials* 1 (2):149–158.
- Guven, S., P. Chen, F. Inci, S. Tasoglu, B. Erkmen, and U. Demirci. 2015. Multiscale assembly for tissue engineering and regenerative medicine. *Trends in Biotechnology* 33 (5):269–279.
- He, X., E. Y. H. Park, A. Fowler, M. L. Yarmush, and M. Toner. 2008. Vitrification by ultra-fast cooling at a low concentration of cryoprotectants in a quartz micro-capillary: A study using murine embryonic stem cells. *Cryobiology* 56 (3):223–232.
- Hopp, B., T. Smausz, N. Kresz, N. Barna, Z. Bor, L. Kolozsvári, D. B. Chrisey, A. Szabó, and A. Nógrádi. 2005. Survival and proliferative ability of various living cell types after laser-induced forward transfer. *Tissue Engineering* 11 (11–12):1817–1823.

- Hurt, A. E., F. Landim-Alvarenga, G. E. Scidel, and E. L. Squires. 2000. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. *Theriogenology* 54 (1):119–128.
- Isachenko, V., M. Montag, E. Isachenko, V. Zaeva, I. Krivokharchenko, R. Shafei, and H. Van der Ven. 2005. Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. *Human Reproduction* 20 (2):492–496.
- Jin, B., F. W. Kleinhaus, and P. Mazur. 2014. Survivals of mouse oocytes approach 100% after vitrification in 3-fold diluted media and ultra-rapid warming by an IR laser pulse. *Cryobiology* 68 (3):419–430.
- Jones, N. 2012. Science in three dimensions: The print revolution. *Nature* 487 (7405):22–23.
- Kattamis, N. T., N. D. McDaniel, S. Bernhard, and C. B. Arnold. 2009. Laser direct write printing of sensitive and robust light emitting organic molecules. *Applied Physics Letters* 94 (10):3. doi:10.1063/1.3098375.
- Khalil, S., and W. Sun. 2007. Biopolymer deposition for freeform fabrication of hydrogel tissue constructs. *Materials Science and Engineering: C* 27 (3):469–478.
- Kim, J. D., J. S. Choi, B. S. Kim, Y. C. Choi, and Y. W. Cho. 2010. Piezoelectric inkjet printing of polymers: Stem cell patterning on polymer substrates. *Polymer* 51 (10):2147–2154.
- Knowlton, S., Y. Cho, X. J. Li, A. Khademhosseini, and S. Tasoglu. 2016a. Utilizing stem cells for three-dimensional neural tissue engineering. *Biomaterials Science* 4 (5):768–784.
- Knowlton, S., A. Joshi, B. Yenilmez, I. T. Ozbolat, C. K. Chua, A. Khademhosseini, and S. Tasoglu. 2016b. Advancing cancer research using bioprinting for tumor-on-a-chip platforms. *International Journal of Bioprinting* 2 (2):111–116.
- Knowlton, S., D. Li, F. Ersoy, Y. K. Cho, and S. Tasoglu. 2016c. Building blocks for bottom-up neural tissue engineering: Tools for in vitro assembly and interrogation of neural circuits. In *Neural Engineering*, 123–144.
- Knowlton, S., S. Onal, C. H. Yu, J. J. Zhao, and S. Tasoglu. 2015b. Bioprinting for cancer research. *Trends in Biotechnology* 33 (9):504–513.
- Knowlton, S. M., M. Sadasivam, and S. Tasoglu. 2015a. Microfluidics for sperm research. *Trends in Biotechnology* 33 (4):221–229.
- Knowlton, S., B. Yenilmez, S. Anand, and S. Tasoglu. 2017. Photocrosslinking-based bioprinting: Examining crosslinking schemes. *Bioprinting* 5:10–18.
- Knowlton, S., B. Yenilmez, and S. Tasoglu. 2016d. Towards single-step biofabrication of organs on a chip via 3D printing. *Trends in Biotechnology* 34 (9):685–688.
- Knowlton, S., S. Anand, T. Shah, and S. Tasoglu. 2017. Bioprinting for Neural Tissue Engineering. *Trends in neurosciences* 41(1):31–46.
- Kuleshova, L. L., and A. Lopata. 2002. Vitrification can be more favorable than slow cooling. *Fertility and Sterility* 78 (3):449–454.
- Kuwayama, M., G. Vajta, S. Ieda, and O. Kato. 2005a. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reproductive Biomedicine Online* 11 (5):608–614.
- Kuwayama, M., G. Vajta, O. Kato, and S. P. Leibo. 2005b. Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive Biomedicine Online* 11 (3):300–308.
- Lane, M., W. B. Schoolcraft, D. K. Gardner, and D. Phil. 1999. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertility and Sterility* 72 (6):1073–1078.
- Lepowsky, E., and S. Tasoglu. 2018. 3D printing for drug manufacturing: A perspective on the future of pharmaceuticals. *International Journal of Bioprinting* 4(1):119–131.
- Luo, Z. Y., S. Güven, I. Gozen, P. Chen, S. Tasoglu, R. M. Anchan, B. Bai, and U. Demirci. 2015. Deformation of a single mouse oocyte in a constricted microfluidic channel. *Microfluidics and Nanofluidics* 19 (4):883–890.

- Malda, J., J. Visser, F. P. Melchels, T. Jüngst, W. E. Hennink, W. J. A. Dhert, J. Groll, and D. W. Huttmacher. 2013. 25th anniversary article: Engineering hydrogels for biofabrication. *Advanced Materials* 25 (36):5011–5028.
- Medeiros, C. M. O., F. Forell, A. T. D. Oliveira, and J. L. Rodrigues. 2002. Current status of sperm cryopreservation: Why isn't it better? *Theriogenology* 57 (1):327–344.
- Mphaphathi, M. L., D. Luseba, B. Sutherland, and T. L. Nedambale. 2012. Comparison of slow freezing and vitrification methods for Venda cockerel's spermatozoa. *Open Journal of Animal Sciences* 2:204.
- Mukaida, T., S. Nakamura, T. Tomiyama, S. Wada, M. Kasai, and K. Takahashi. 2001. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertility and Sterility* 76 (3):618–620.
- Muradoglu, M., and S. Tasoglu. 2010. A front-tracking method for computational modeling of impact and spreading of viscous droplets on solid walls. *Computers & Fluids* 39 (4):615–625.
- Murphy, S. V., and A. Atala. 2014. 3D bioprinting of tissues and organs. *Nature Biotechnology* 32 (8):773–785.
- Nahmias, Y., and D. J. Odde. 2006. Micropatterning of living cells by laser-guided direct writing: Application to fabrication of hepatic-endothelial sinusoid-like structures. *Nature Protocols* 1 (5):2288–2296. doi:10.1038/nprot.2006.386.
- Nahmias, Y., R. E. Schwartz, C. M. Verfaillie, and D. J. Odde. 2005. Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnology and Bioengineering* 92 (2):129–136. doi:10.1002/bit.20585.
- Nakamura, M., A. Kobayashi, F. Takagi, A. Watanabe, Y. Hiruma, K. Ohuchi, Y. Iwasaki, M. Horie, I. Morita, and S. Takatani. 2005. Biocompatible inkjet printing technique for designed seeding of individual living cells. *Tissue Engineering* 11 (11–12):1658–1666. doi:10.1089/ten.2005.11.1658.
- Odde, D. J., and M. J. Renn. 1999. Laser-guided direct writing for applications in biotechnology. *Trends in Biotechnology* 17 (10):385–389.
- Panagopoulou, M., N. Nikolettos, V. Dala, E. Tsakos, K. Charalabopoulos, and B. Asimakopoulos. 2013. A comparison between two vitrification devices: Plastic blade and McGill cryoleaf. *Human Genetics & Embryology* 3 (102).
- Phillippi, J. A., E. Miller, L. Weiss, J. Huard, A. Waggoner, and P. Campbell. 2008. Microenvironments engineered by inkjet bioprinting spatially direct adult stem cells toward muscle- and bone-like subpopulations. *Stem Cells* 26 (1):127–134.
- Pyne, D. G., J. Liu, M. Abdelgawad, and Y. Sun. 2014. Digital microfluidic processing of mammalian embryos for vitrification. *PLoS One* 9 (9):e108128.
- Reubinoff, B. E., M. F. Pera, G. Vajta, and A. O. Trounson. 2001. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. *Human Reproduction* 16 (10):2187–2194.
- Samot, J., S. Moon, L. Shao, X. Zhang, F. Xu, Y. S. Song, H. O. Keles, L. Matloff, J. Markel, and U. Demirci. 2011. Blood banking in living droplets. *PLoS One* 6 (3):e17530.
- Scholz, E. C. 2012. The problem of contamination: Open vs. closed vs. semi-closed vitrification systems. INTECH Open Access Publisher.
- Selman, H. 2005. Vitrification versus conventional cryopreservation technique. *Middle East Fertility Society Journal* 10 (3):207–209.
- Sheikhi, M., K. Hultén, B. Niklasson, M. Lundqvist, and O. Hovatta. 2013. Preservation of human ovarian follicles within tissue frozen by vitrification in a xeno-free closed system using only ethylene glycol as a permeating cryoprotectant. *Fertility and Sterility* 100 (1):170–177.
- Shi, M., K. Ling, K. W. Yong, Y. Li, S. Feng, X. Zhang, B. Pingguan-Murphy, T. J. Lu, and F. Xu. 2015. High-throughput non-contact vitrification of cell-laden droplets based on cell printing. *Scientific Reports* 5:17928.

- Song, Y. S., D. Adler, F. Xu, E. Kayaalp, A. Nureddin, R. M. Anchan, R. L. Maas, and U. Demirci. 2010. Vitrification and levitation of a liquid droplet on liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America* 107 (10):4596–4600.
- Song, Y. S., S. J. Moon, L. Hulli, S. K. Hasan, E. Kayaalp, and U. Demirci. 2009. Microfluidics for cryopreservation. *Lab on a Chip* 9 (13):1874–1881.
- Takahashi, K., T. Mukaida, T. Goto, and C. Oka. 2005. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertility and Sterility* 84 (1):88–92.
- Tasoglu, S., and U. Demirci. 2013. Bioprinting for stem cell research. *Trends in Biotechnology* 31 (1):10–19.
- Tasoglu, S., E. Diller, S. Guven, M. Sitti, and U. Demirci. 2014a. Untethered micro-robotic coding of three-dimensional material composition. *Nature Communications* 5:3124.
- Tasoglu, S., U. A. Gurkan, S. Q. Wang, and U. Demirci. 2013a. Manipulating biological agents and cells in micro-scale volumes for applications in medicine. *Chemical Society Reviews* 42 (13):5788–5808.
- Tasoglu, S., G. Kaynak, A. J. Szeri, U. Demirci, and M. Muradoglu. 2010. Impact of a compound droplet on a flat surface: A model for single cell epitaxy. *Physics of Fluids* 22 (8):082103.
- Tasoglu, S., H. Safaee, X. Zhang, J. L. Kingsley, P. N. Catalano, U. A. Gurkan, A. Nureddin, E. Kayaalp, R. M. Anchan, and R. L. Maas. 2013b. Exhaustion of racing sperm in nature-mimicking microfluidic channels during sorting. *Small* 9 (20):3374–3384.
- Tasoglu, S., C. H. Yu, H. I. Gungordu, S. Guven, T. Vural, and U. Demirci. 2014b. Guided and magnetic self-assembly of tunable magnetoceptive gels. *Nature Communications* 5:4702.
- Tavukcuoglu, S., T. Al-Azawi, A. A. Khaki, and S. Al-Hasani. 2012. Is vitrification standard method of cryopreservation. *Middle East Fertility Society Journal* 17 (3):152–156. doi:10.1016/j.mefs.2012.07.007.
- Tsai, S., and C. Lin. 2012. Advantages and applications of cryopreservation in fisheries science. *Brazilian Archives of Biology and Technology* 55 (3):425–434.
- Valbuena, D., M. E. Póo, C. Aguilar-Gallardo, S. Martinez, A. C. Cobo, A. Pellicer, and C. Simón. 2012. Comparison of cryotip vs. cryotop for mouse and human blastomere vitrification. *Fertility and Sterility* 97 (1):209–217.
- Wang, X., A. A. Mäkitie, J. Partanen, J. Tuomi, K. S. Paloheimo, and M. Yliperttula. 2013. *The Integrations of Biomaterials and Rapid Prototyping Techniques for Intelligent Manufacturing of Complex Organs*. INTECH Open Access Publisher, London, UK.
- Wang, X., X. Zhang, Y. Qin, D. Hao, and H. Shi. 2012. Outcomes of day 3 embryo transfer with vitrification using Cryoleaf: A 3-year follow-up study. *Journal of Assisted Reproduction and Genetics* 29 (9):883–889.
- Xu, F., S. Moon, X. Zhang, L. Shao, Y. S. Song, and U. Demirci. 2010. Multi-scale heat and mass transfer modelling of cell and tissue cryopreservation. *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences* 368 (1912):561–583.
- Xu, T., C. A. Gregory, P. Molnar, X. Cui, S. Jalota, S. B. Bhaduri, and T. Boland. 2006. Viability and electrophysiology of neural cell structures generated by the inkjet printing method. *Biomaterials* 27 (19):3580–3588.
- Xu, T., J. Jin, C. Gregory, J. J. Hickman, and T. Boland. 2005. Inkjet printing of viable mammalian cells. *Biomaterials* 26 (1):93–99.
- Yong, K. W., W. K. Z. Wan Safwani, F. Xu, W. Abas, J. R. Choi, and B. Pinguang-Murphy. 2015. Cryopreservation of human mesenchymal stem cells for clinical applications: Current methods and challenges. *Biopreservation and Biobanking* 13 (4):231–239.

- Zhang, X., P. N. Catalano, U. A. Gurkan, I. Khimji, and U. Demirci. 2011. Emerging technologies in medical applications of minimum volume vitrification. *Nanomedicine* 6 (6):1115–1129.
- Zhang, X., I. Khimji, L. Shao, H. Safaee, K. Desai, H. O. Keles, U. A. Gurkan, E. Kayaalp, A. Nureddin, and R. M. Anchan. 2012. Nanoliter droplet vitrification for oocyte cryopreservation. *Nanomedicine* 7 (4):553–564.
- Zhou, X., Z. Liu, X. M. Liang, Z. Shu, P. Du, and D. Gao. 2013. Theoretical investigations of a novel microfluidic cooling/warming system for cell vitrification cryopreservation. *International Journal of Heat and Mass Transfer* 65:381–388.
- Zou, Y., T. Yin, S. Chen, J. Yang, and W. Huang. 2013. On-chip cryopreservation: A novel method for ultra-rapid cryoprotectant-free cryopreservation of small amounts of human spermatozoa. *PloS One* 8 (4):e61593.



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Chapter 15 4D bioprinting

Mechanism and applications

Qingzhen Yang, Yuan Ji,
and Feng Xu

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15.1 Introduction: Evolution from 3D bioprinting to 4D bioprinting

Nowadays, 3D printing has become well-known to researchers and the public due to its widespread applications in biomedical engineering [1–3] and other fields [4,5]. In such a technique, one or multiple materials are printed through nozzles, and by controlling the shape of each individual layer, a 3D construct with no (or little) spatial restrictions can be implemented. Recently, 3D printing is employed to print live cells together with biocompatible materials, which could form into complex 3D tissues *in vitro* (termed as 3D bioprinting) [6], with the potential applications to address the need for regeneration of functional tissues and/or organs that are suitable for drug screening or even direct transplantation and other biomedical applications [6,7].

In spite of numerous advantages and potential applications, there is one major drawback of the 3D bioprinting: it considers only the initial shapes of printed constructs, or in other words, they are static and inanimate. To overcome this hurdle, a novel technique, termed as *4D bioprinting*, is emerging. In 4D bioprinting, an extra dimension *time* is introduced to 3D bioprinting. Here *time* is not about the time-consumption for printing the 3D construct, but actually it refers to the fact that the 3D-printed living cellular constructs or biocompatible materials continue evolving with time. Here in this chapter, we categorize the 4D bioprinting into two types (Figure 15.1 for the details). The first type refers to the bioprinting of *smart* materials, which usually utilize the responsive materials that are able to reshape or have the functionality to deform according to the external stimulus. This type of 4D bioprinting is very similar with some well-known phenomena, for example, self-folding, self-assembly, and self-disassembly [8–10]. The second one is based on the development and/or maturation of bioprinted constructs with/without external stimulus after printing. It is known that 3D bioprinting can construct *microtissue* with bioink (e.g., cell-laden microgels) through a layer-by-layer printing approach, where cells are situated in native-mimicking 3D microenvironment in the printed construct. In this condition, the printed *microtissue* can undergo development/maturation *via* cellular coating, self-organization, and matrix deposition to form functional tissue after printing with time [11,12].

As a promising technique, 4D bioprinting could be a powerful tool for finding solutions to medical problems, and may find numerous applications for it in biomedical engineering. However it is not well known or fully understood to the researchers. In the following sections, we present recent advances in 4D-bioprinting approaches together with the potential applications in biomedical fields. Special interests are focused on the mechanism of 4D bioprinting and its applications in drug screening and tissue engineering (TE). Besides, the current roadblocks to 4D bioprinting together with possible solutions are also discussed to provide the future directions.

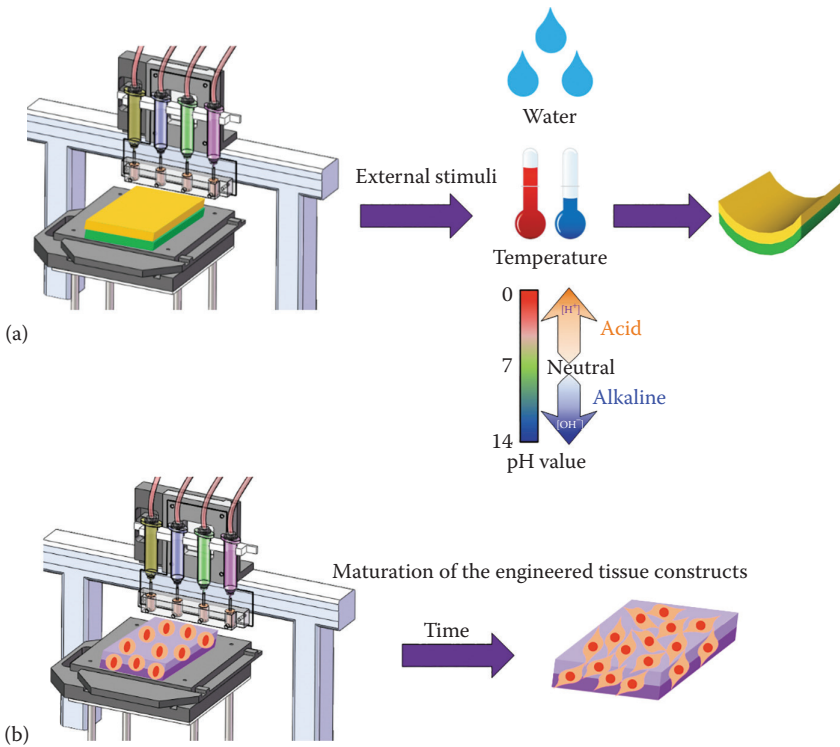


Figure 15.1 Illustration of 4D bioprinting. (a) The first type of 4D bioprinting, which is based on deformation of *smart* materials. The bioprinted constructs would self-fold as a response of external stimuli (e.g., water, temperature, or pH value). (b) The second type of 4D bioprinting, which is based on the development and/or maturation of the bioprinted cells and/or tissues.

15.2 4D bioprinting based on *smart* materials

With the development of material science, more and more materials (e.g., lipids [13,14], polymers [15,16]) have been made bioprintable. For the first type of 4D bioprinting, the *smart* materials are printed that are able to reshape or change their function due to the external stimuli, such as temperature [15,17,18], water [9,13,14], pH value [19], and so on. After imposing the external stimulus, the bioprinted 3D construct would deform spontaneously or transform into a different structure. By utilizing the 3D-bioprinting device, the *smart* materials can be printed into a 3D construct (e.g., scaffold), which has the capability to reshape or self-fold according to the external stimulus. Thus, introducing this fourth dimension will help to contribute greatly to the fabrication of complex construct and

temporally controllable deformation after printing. These may find plenty of applications in engineering the functional tissues or even organs.

As stated before, the materials in 4D printing are required to adapt their shapes based on the environment stimuli. According to its deforming mechanism, the 4D printing can be categorized into some types, such as deformation due to thermal stimulus, light, water absorption, surface tension, cell traction, or their combination. Subsequently, we would describe each type in detail.

15.2.1 Deformation due to water absorption

In this section, the hydrophilic material is introduced, which could be 3D-printed but later time transforms into another shape or configuration. Hydrophilic materials and structures deform due to the hygroscopic effect, where the material swells in a temporally and spatially dependent manner when immersed in water. It has been demonstrated that the hydrophilic polymer can expand 150% when it encounters water [9]. If well designed, the structure made of hydrophilic material can be precisely folded into a new configuration. Tibbitts et al. have developed several prototypes, for instance, the printed strand that could self-fold into the designed pattern when immersed into water (Figure 15.2a). In their experiment, a strand with a length of 30 cm was printed, which contains both hydrophilic and rigid materials. The rigid part performs as angle limiters during the folding process. When this strand was immersed in water, the hydrophilic materials swelled, forcing the rigid materials to deform. Once the rigid materials hit the neighboring part, the deformation would stop and thus the entire part achieved its final steady state. If carefully designed, the strand that was initially straight can transform into the letters *MIT*. Another structure printed by both rigid and hydrophilic materials demonstrated the transformation of two-dimensional (2D) planar structure. In this demonstration, an initially flat 2D plane structure was printed first, representing the six unfolded surfaces of a cube. For each junction of two adjacent surfaces, the aforementioned configuration (Figure 15.2a) was printed and served as a joint. The main function is to stop the surface from folding and ensure that it reached into the final-state configuration. Immersed into water, the structure folded into a closed-surface cube. The above-mentioned examples demonstrate that the hydrosensitive materials could deform according to the stimulus of water and it can be implemented for both one-dimensional and 2D structure [9].

Another commonly used hydrosensitive material is hydrogel, and its deformation is based on the fact that different compartments of hydrogel scaffold have different water sorption. Using such hydrogels, the bioprinted scaffolds can self-deform in a programmable manner through water sorption. For instance, Jamal et al. printed photopatterned polyethylene glycol (PEG) of two different molecular weights into a two-layered structure, which could self-fold when it encountered water (Figure 15.2b) [16]. As the two PEG bilayers have different absorption in

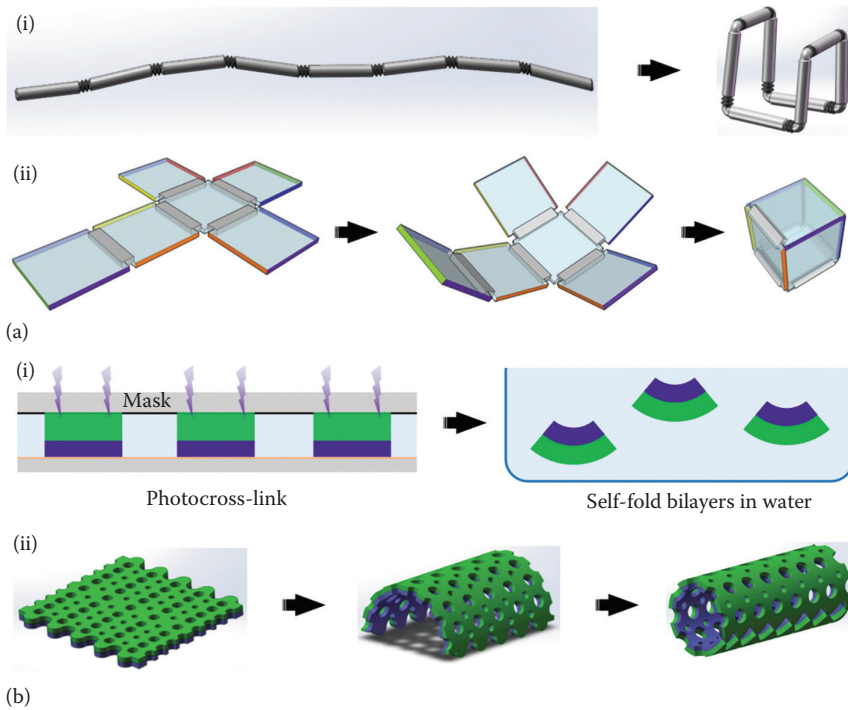


Figure 15.2 4D bioprinting due to the deformation of water-absorbable *smart* materials. (a) 4D-printed rigid materials: (i) Deformation of a single strand when it is immersed in water and (ii) deformation of two-dimensional flat plane. (b) Bioprinted soft materials in response to water: (i) Schematic of fabrication of the PEG bilayers and (ii) schematic illustration of the self-folding of an initially flat PEG bilayer.

aqueous solutions, the bioprinted scaffold would deform spontaneously, resulting in the curvature of the scaffold. Besides, cells can be encapsulated in the PEG bilayers. When immersed in water, the cell-laden scaffold can deform into a cylinder whose radius is controllable. In this method, the cell's viability is very high after an 8-week culture indicating that it has no detrimental effect on the encapsulated cells. Actually the shape of bilayer PEG scaffold can be customized. With careful design, different microscale geometries can be obtained using this technique.

The aqueous droplets network can also deform in water. Villar et al. printed heterogeneous picoliter aqueous droplets into a 3D construct [13,14]. After printing, these aqueous droplets can self-assemble into a cohesive structure with cooperating compartments. Moreover, the droplet network can be functionalized with membrane proteins. Villar et al. have demonstrated that the network is able to allow rapid electrical communication along a predesigned path. Besides, the

droplets network can be programmed into a construct with osmolarity gradient and such a construct could self-fold in a controllable manner with stimulus of water [14]. On the other hand, the aqueous droplets can release the encapsulated contents by tuning the temperature or pH value [13].

15.2.2 Deformation due to thermal stimulation

The most frequently used materials in 4D printing are thermal-sensitive, and the deforming process is shown in Figure 15.3a. A 3D printer is used to print the soft composites, which consist of thermal-sensitive fibers reinforcing an elastomeric matrix. The fibers, by the way, are distributed at the bottom of the matrix. After printing, the entire structure is heated to a high temperature T_H at which

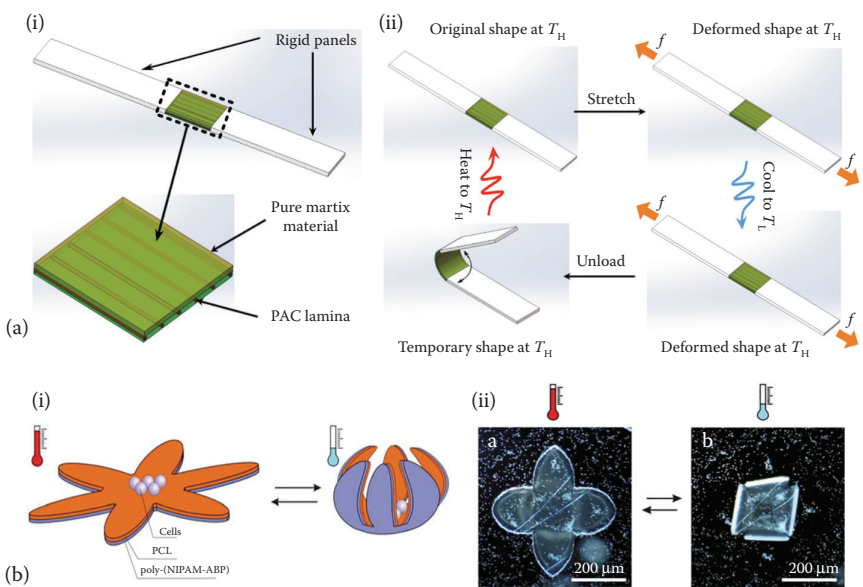


Figure 15.3 4D bioprinting due to the deformation of thermal-responsive materials. (a) 4D-printed rigid materials that are thermoresponsive: (i) The composition of the thermoresponsive object and (ii) the deformation process of this thermoresponsive material. (b) Bioprinted soft materials in response to temperature: (i) Schematic of the folding star-shaped polymer bilayer. Swelling of the thermoresponsive hydrogel layer at lower temperature increases stress in the film that results in bending of the star arms and folding. (Reproduced from Stoychev, G. et al. *Soft Matter*, 7, 3277–3279, 2011. With permission.) and (ii) encapsulation of yeast cells inside the thermoresponsive capsules. Yeast cells are adsorbed on the polymer bilayer at an elevated temperature. Cooling leads to swelling of the thermoresponsive polymer and folding of the capsules. Further heating results in unfolding of the capsules and release of the cells.

the fiber is on its rubbery solid state (i.e., soft state). Meanwhile an external force is imposed at the two ends and the elastic energy is stored inside. Then the temperature is decreased to T_L with the external force maintained and the fiber becomes glassy state (i.e., stiff state). At this very moment, this structure would bend up after releasing the force. If heated to the temperature T_H , the film can recover to flat again. During the temperature cycle, the fibers undergo a glass transition, which enables their usage as the switchable segment in the composite. The elastomer, however, behaves as a rubbery solid during the whole process. In this figure, a simple geometry is shown as an illustration; actually it could be designed into a complex shape. Adapting this method, Ge et al. printed the composite *via* a 3D-multimaterial polymer printer. The self-folding hinges and even self-encapsulating boxes were obtained after thermal stimulus [8,20].

Hydrogels that can sense and respond to external temperature were also used for 4D printing. These hydrogels commonly undergo a volume phase transition (swelling/contracting) following a lower critical solution temperature (LCST). For hydrogels, the temperature changes their water-absorption ability and thus causes the deformation. For instance, a thermal-responsive hydrogel poly (*N*-isopropylacrylamide) (pNIPAM) becomes hydrophobic expelling water and loss of 90% of its mass when the temperature is above LCST. On the other hand, it is hydrophilic and absorbs water if the temperature is below LCST [21]. Besides, the phase-transition temperature of pNIPAM is close to physiological temperature and thus can be widely used in biomedical engineering. For example, PNIPAAm and poly(ϵ -caprolactone) (PCL), water-insoluble polymers, were coprinted into a bilayered construct, which can deform according to the environmental temperature. Stoychev et al. used such a construct for yeast cell encapsulation and release (Figure 15.3b) [22]. Another example is printing ceramic powder (Al_2O_3) and poly(acrylic acid)–PNIPAAm together. For this printed construct, it could undergo a controllable sol–gel transition by tuning the environmental temperature, with potential applications in TE and drug delivery [17]. The major merits of hydrogels are that they are 3D-printable and biocompatible. Up to the present, several kinds of thermal-sensitive polymers have been successfully developed and formed hydrogels *via* 4D printing [23]. For instance, thermosensitive polymers coupled with photocross-linkable groups were used to form biocompatible and biodegradable hydrogels, emerging as an interesting candidate for biomedical applications [24–27].

15.2.3 Deformation due to pH value

The pH-sensitive materials can deform according to the pH value of environment. A typical example is hydrogel composed of pendant-acidic (e.g., carboxylic and sulfonic acids) or -basic (e.g., ammonium salts) groups that could accept or release protons when changing the environmental pH value [28,29]. The dissociation of polymer will sense and respond to the changes of environmental pH,

regulating the ion concentration within the hydrogel network. For instance, poly (*N*, *N'*-diethylaminoethyl methacrylate) (PDEAEM) tends to become ionized at a low pH value. However, poly (acrylic acid) (PAA) becomes ionized when the pH is high [19,30]. Cationic polyelectrolytes (e.g., PDEAEM) dissolve more or swell more if it is cross-linked at low pH, whereas polyanions (e.g., PAA) dissolve more at high pH. The variation of ion concentration causes the absorbing or releasing of water and thus the deformation of hydrogel. This deformation mechanism is very similar to these thermal-sensitive hydrogels except the trigger in this case is pH value.

Besides the aforementioned materials and deforming mechanism, there are some other smart materials that could transform their shapes according to the environment stimulus. Although these materials have not been printed at the present, we incorporate them in this chapter as they are highly related to self-deformable structures and can be potentially used in the 4D printing.

15.2.4 Deformation due to light

Some materials are responsive to light and could be utilized in 4D printing. According to their deformation mechanism, these materials are categorized into three types. The first one is the materials that tend to contract spontaneously once exposed in light with certain wavelength; one representative example is the liquid crystalline elastomers. The light causes the cooperative movement of the molecules and thus the collapse of the alignment order, leading to a considerable contraction. As illustrated in [Figure 15.4a](#), after exposed in light the film tends to fold up as the upper surface absorbs more light than the bottom one and thus shrinks more significantly. Besides, the curling direction is controllable by changing the light's polarization angle [31]. The film would recover when the light is switched off or exposed in light with a different wavelength. Li et al. tested the contraction fraction of an azo chromophore film (a liquid crystalline elastomer) and found that the value can achieve up to 18% when subjected to the irradiation [32]. The second type refers to the materials that could change their molecular backbone in light. These materials cannot contract spontaneously but have to combine with an external stress in a similar manner as the thermal-responsive materials. The light breaks the molecular chains in the network and releases the internal stress. Ryu et al. used this photo-induced stress relaxation method (sometimes referred as photo-origami) and obtained some complex structures by bending and folding [33]. Another light-activated material uses photothermal mechanism, the function of light in this case merely provides the heat source [34]. Although it is triggered by light, essentially it is heat that causes the subsequent deformation. Light-induced self-deformation is particularly attractive because it provides a precise control of the location where the stimulus is applied; moreover, it enables the noncontact actuation and thus avoids the unfavorable damage of tissue when a smart implant is activated in the body.

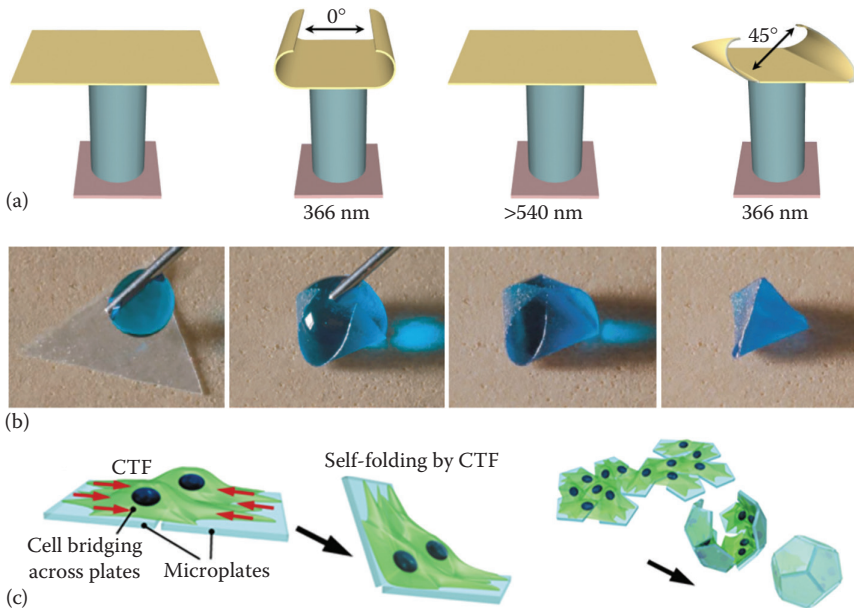


Figure 15.4 4D bioprinting due to the material deformation under external stimuli. (a) Material deformation due to light and the deformation can be controlled by the light wavelength. It deforms under the light with a wavelength of 366 nm and retreats back to flat if the wavelength is longer than 540 nm. (b) Deformation due to surface tension. A liquid droplet is placed on a thin solid film and it would deform due to the surface tension. With the evaporation of the liquid, the film would bend over until it is completely wrapped. (Reproduced from Py, C. et al., *Eur Phys J Spec Top*, 166, 67–71, 2009. With permission.) (c) Deformation due to cell traction force (CTF). Some cells have a trend of spontaneous contraction, thus the cells printed on microplates would cause the rotation around the hinge. By a careful design, a closed object can be obtained. (Reproduced from Kuribayashi-Shigetomi, K. et al., *PLoS One*, 7, e51085, 2012. With permission.)

15.2.5 Deformation due to surface tension

Surface tension-caused deformation, which sometimes is referred as capillary origami, can be potentially used as 4D printing. Py et al. first discovered such a phenomenon by showing that an elastic thin sheet can be folded up spontaneously by the surface tensions at the contact line when it is in contacted with a droplet; this droplet meanwhile evolves into a 3D shape [35]. The typical process of capillary origami is shown in Figure 15.4b, first a droplet is deposited on a triangular-shaped PDMS film while ensuring that the droplet arrives to the corners of the film. As the droplet evaporates, the initial flat film becomes unstable

and it starts to deform and wraps up gradually. This film could get completely closed and confine some liquid inside, thus forming a 3D pyramid structure. The final encapsulated 3D shape could be changed by tuning the initial geometry of the film. Up to present, the pyramidal, spherical, cubical, and triangular structures have been successfully obtained by such a capillary origami technique [36,37]. For a high throughput, the droplets can be 3D-printed on an array of PDMS films, which would deform spontaneously. One potential application of this capillary origami-based 4D printing is fabricating different-shaped hydrogel particles that are useful in TE.

15.2.6 Deformation due to cell traction

To the present, 3D printing enables the mixing of living cells with biocompatible materials to form the bioink, and then the bioink is loaded and printed to form cell-laden microstructures. It is known that some cells tend to contract spontaneously and exert a contractile force [38] (sometimes is referred as cell traction force [CTF]). Such a force is generated due to the actomyosin interactions and actin polymerization. In this section, deformation over time of 3D cell-laden microstructures driven by CTF is introduced. As illustrated in [Figure 15.4c](#), the cells were cultured on two microplates. After 24 ~ 48 hours culture, the microplates were slightly lifted at their edges with the purpose of detaching the microplates from substrate. Then the microplates were pulled by the CTF originating from the cells cultured on the plates. Microplates kept rotating until it get contacted with the adjacent one. In contrast, if multiple cells, rather than one type, are cultured on the microplates, the cocultured cells contact with each other. For this case, microplates would also fold up. However, the main driving force is the CTF that are exerted by cell–matrix interaction rather than the cell traction itself, this interaction tends to reinforce the folding force and thus leads to a large deformation [28]. Kuribayashi–Shigetomi et al. [38] used the living cells and the patterned 2D microplates to fabricate various deformable 3D cell-laden constructs. Three deformable and hollow microstructures such as cube, dodecahedron, and cylindrical helical tube were successfully produced with NIH/3T3 cells. For the cylindrical tube, the initially flat microplates were folded up along its diagonal lines. Furthermore, normal human umbilical vein endothelial cells (HUVECs) and bovine carotid artery endothelial cells were also used to produce cylindrical tubes with the purpose of mimicking vessel-like structures.

15.2.7 Others

Recent developments enabled the deformation of bioprinted constructs through other external forces, such as electrical, magnetic, and pneumatical force. For instance, Shao's group used electric field as the driving force to

assemble micro-/nanoparticles in a well-controlled pattern [39–44]. Recently, the deformation induced by magnetic field could be used to control the orientation of anisotropic building blocks (e.g., microparticles) during or after the printing process [45]. Kokkinis et al. printed inks that were loaded with magnetic stiff platelets and then imposed a low magnetic field to control the particle orientation. Multimaterial dispensers and a two-component mixing unit enable an extra control over the local composition of the printed material. Such a design opens the way toward the manufacturing of functional heterogeneous materials with exquisite microstructural features, which can highly mimic complicated biological constructs in nature.

15.3 4D bioprinting based on the maturation of engineered tissue constructs

For the first type of 4D bioprinting, the bioprinted materials can deform as a response to the external stimulus, which can be used to create construct with high complexity. This goal can also be implemented by the self-organization of cells, which are encapsulated in or coated on the bioprinted scaffolds [46]. 3D bioprinting is capable to simultaneously print biocompatible materials, living cells together with growth factors at precisely controlled locations with the purpose of constructing the native tissue [47]. However, the printed tissue constructs are usually not suitable for direct use (e.g., drug screening platform *in vitro*, or direct transplantation) as the printed constructs are not fully developed or matured. Sometimes, post-printing processes such as cell coating, cell self-organization, matrix deposition, and cell alignment are needed to promote the development or maturation of the printed cellular constructs. This is referred as the second type of 4D bioprinting, and it provides an alternative to fabricate engineered tissue constructs with functionality that may be used in practical applications.

15.3.1 Cell coating

To make the vascular graft mature, a commonly adopted method is to coat the endothelial cell (EC) layer in the lumen of graft [48,49]. The coated EC layer with the capability in reducing thrombosis (static aggregation of blood factors) can prevent the blocking and can ensure the patency of the printed vascular grafts for a long time. After removing the aqueous fugitive ink, what is left in the engineered tissue constructs is tubular vasculature-shaped structure. Then, the bioink encapsulating human umbilical vein endothelial cells (HUVEC) was printed into the vasculature structure. After 48 hours of incubation, a confluent HUVEC cellular layer has been obtained on the lumen of the printed vasculature. The HUVEC-coated vascular walls serve as both the source and sink for the soluble factors and advance the maturation of the printed *microtissue* [50].

15.3.2 Cell self-organization

Cell self-organization was also adopted in 4D bioprinting to construct tubular grafts with the bioinks in the form of cellular toroids that contain human ovarian granulosa cells [51]. The toroids were printed on a small diameter capillary tube to form a tubular structure. After printing, the cell self-organization process underwent in each toroid over a period of 72 h, leading to the closing of small gaps between each toroid and forming a connective tubular graft [52]. The living cells within the printed tubular graft communicated with each other *via* different kinds of signaling molecules and adjust their positions within the matrix to form the connective and consistent tubular grafts (Figure 15.5). Process as cell self-organization will promote the maturation of the printed tissue to acquire the necessary mechanical properties for implantation.

15.3.3 Matrix deposition

Similar to cell coating and self-organization, matrix deposition from the cells within the bioprinted tissue construct can also promote construct maturation in 4D bioprinting. Phase-changing hydrogels are the commonly used bioink in 3D bioprinting due to the merits such as easy processability and good biocompatibility with encapsulated cells. However, the inherent weakness of hydrogels such as quick degradation will hinder the final strength of the tissue-engineered matrix and threaten the integrity of the printed construct. To address this, human mesenchymal stem cells (hMSCs) were seeded onto bioprinted tissue construct

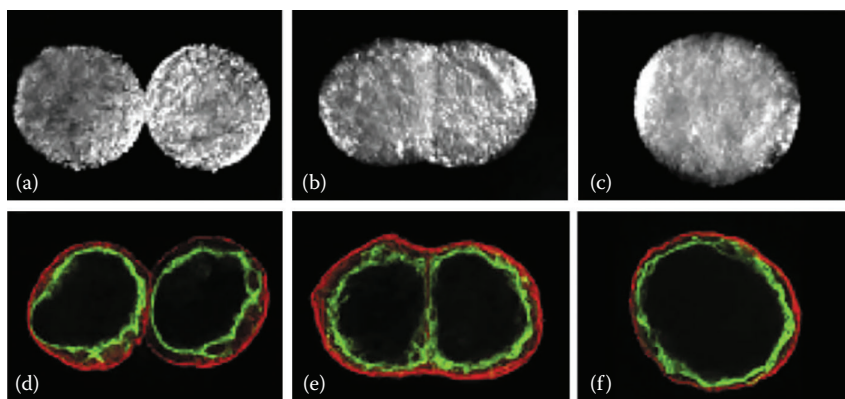


Figure 15.5 4D bioprinting based on the maturation of cells/tissues. These images show the self-assembled process of vascular tissue spheroids, which were bioprinted and then cultured in hanging drop. The dynamic evolution process was recorded by optical images (a–c) and confocal images (d–f). (Reproduced from Mironov, V. et al., *Biomaterials*, 30, 2164–2174, 2009. With permission.)

with grid pattern [22]. The degradation time of the tissue construct in media was prolonged from 2 days to more than 2 weeks due to the matrix deposition from hMSCs. Thanks to the matrix deposition from hMSCs, the grid-like pattern was maintained and with enough robustness for handling. This emphasizes the importance of optimizing bioink degradation with matrix deposition and remodeling by cells for engineering functional tissues [53]. As demonstrated earlier, with the involvement of time scale in 4D bioprinting, the printed tissue constructs become mature and show similarity as native functional tissues.

15.3.4 Cell alignment

Some cells—for example, cardiomyocyte, neural cells, and vascular endothelial cells—in native tissues tend to align in some certain direction. Cell alignment, which refers to spatial orientation of cells, significantly affects the pattern formation during tissue development, maturation, and regeneration growth [54]. Cell alignment in natural tissues is generally accompanied by cells' proliferation, differentiation, and other cells behavior. However, the bioprinting cells usually show a random arrangement and thus a post-process sometimes is needed to ensure the cells alignment. Li et al. implemented cell alignment by imposing directional forces through a magnetically actuated and noncontact method [55,56]. They have found that the cells' behaviors including proliferation, spreading, and alignment can be significantly affected. This noncontact magnetic force would induce muscle myofiber formation. Another alternative is printing cells on the substrate with micro-/nanostructures. It has been found that microgrooves on substrate can guide cardiomyocyte evolved into anisotropic engineered tissues with time [57].

15.4 Applications of 4D bioprinting

Much effort has been made for TE, in which cells are seeded onto a biocompatible scaffold or encapsulated in a hydrogel, enabling cells to populate and create their own extracellular matrix (ECM) [6]. Several thin or avascular tissues, such as bladder, cartilage, and skin, have been successfully implemented *in vitro*. Nonetheless, their practical applications with regard to regeneration of functional tissues are still limited due to the lack of accurate control of the cellular architecture and ECM distribution in large-scaled scaffolds. The emergency of 4D bioprinting provides a powerful tool for biomedical engineering such as drug delivery and tissue regeneration due to their remarkable advantages: (1) 4D bioprinting enables 3D complex tissue constructs that could self-deform as a response to the external stimulus and (2) 4D bioprinting enables the development/maturation of cells that are encapsulated in or coated on the biocompatible scaffolds, making the bioprinted cellular constructs functional as native tissues. Notably, the

self-organization of cells seems more important during the 4D-bioprinting process, which is the fundamental biological principle of tissue/organ formation *in vitro*. In this section, several examples based on 4D bioprinting, for TE and drug delivery, are presented.

15.4.1 Applications of 4D bioprinting in tissue engineering

Recent advances in our understanding of the structures of blood vessels and the availability of sophisticated technique of 4D bioprinting have significantly changed the landscape of potential strategies for fabricating biomimetic blood vessels *in vitro*. Self-folding polymers, which can encapsulate different types of cells and form multilayered tubular structures in the presence of water can be used to fabricate blood vessels. An alternative to construct vasculature through 4D bioprinting is to take advantage of the natural self-organization abilities of the cells, which are coated on or encapsulated during the bioprinting process [46].

Vascularization is an important issue in TE that needs to be addressed because as it is necessary for engineered functional tissues with a scale larger than 100–200 μm (the limit of diffusion in tissue) [58,59]. Blood vessel is the main channel to supply cells with nutrients and oxygen and to remove waste products, which is important to maintain the organ activity and functionality. Now, 4D printing provides an alternative solution to address this problem. Through layer-by-layer printing, the cell-laden hydrogels can be printed into tubular structure such as the vasculature [60]. Then stimulated by maturation factors, the bioprinted cells rapidly matured and showed its biological characteristics. For example, Hong et al. have printed multiple cell types, including fibroblasts, MSCs, and ECs, together with hydrogel into a pattern [61]. Then after 16 days of culture, the cell migration and aggregation occurred, which eventually formed a vascular structure as evidenced by expression of endothelial-specific genes. In the experiment, several factors including growth factors, ECS–MSCs interactions, ECM components, and mechanical factors could influence the shape and functionality of the vasculature structure [3,4]. Another emerging 4D-printing technique mentioned earlier is to print tens of thousands of picoliter aqueous droplets that are joined by lipid bilayers, which would form a cohesive material with cooperating compartments [14]. 3D tissue-like structures can be easily built with heterologous droplets in software-defined arrangements. The networks can also be programmed by osmolarity gradients to fold into otherwise unattainable designed structures, which hold great potential to fabricate vascular-like tissue constructs. One major challenge of these systems is the incapability to form large vascular structure due to the limited diffusion properties of the hydrogels. In addition, the removal of the supporting scaffold still remains challenging for vascularization *in vitro*.

Different from this strategy, which uses residual stress and elastic modulus, another method enables self-folding through a tangential tension exerted by cells

to the ECM or the underlying layer, known as CTF. The geometry of the patterned 2D microplates folded up along the diagonal lines through CTF. Then the diameter of the tube can be adjusted by tuning the angle of the diagonal lines. Cylindrical tubes were constructed using normal HUVECs and bovine carotid artery endothelial cells to mimic vascular structures [38].

Agarose rods fabricated by bioprinting were also used as the filling material to control the size and shape of vascular tubes [60,62,63]. In this method, blood vessels with different geometry (e.g., double layer and branching) have been successfully produced (Figure 15.6). Printed human skin fibroblasts and human vein smooth muscle cells in agarose have assembled into a vascular-like tube through cell fusion and maturation. However, one disadvantage of

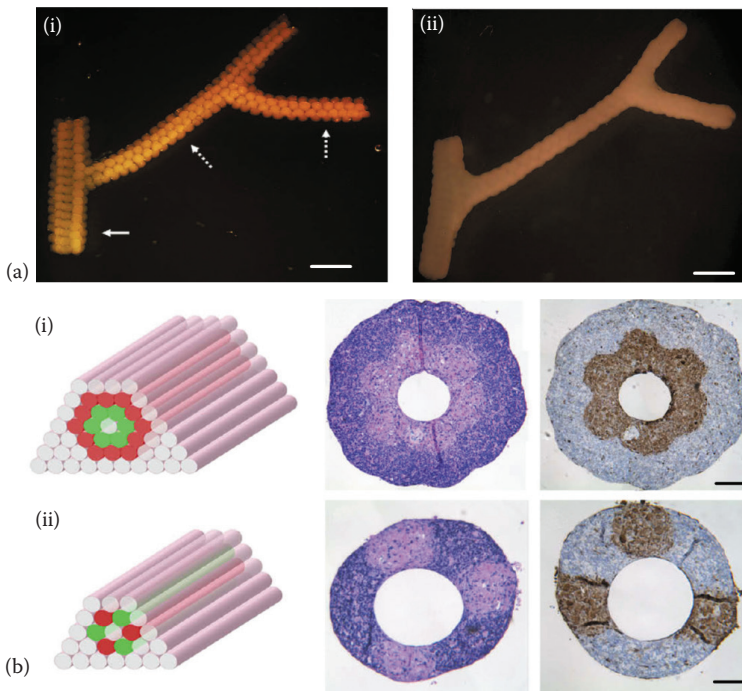


Figure 15.6 Applications of 4D bioprinting for vascularization. (a) A branch-shaped structure fabricated by 4D bioprinting: (i) The bioprinted cellular spheroids patterned in a branch shape and (ii) after 6 days of deposition, the cells were fused and formed to a branched construct. Scale bars, 1000 μm . (Reproduced from Norotte, C. et al., *Biomaterials*, 30, 5910–5917, 2009. With permission.) (b) Coculture of human skin fibroblasts and human vein smooth muscle cells. After 3 days of fusion, the co-cultured cells formed into specific patterns. Tubular vascular structures were generated. Scale bar: 500 μm . (Reproduced from Norotte, C. et al., *Biomaterials*, 30, 5910–5917, 2009. With permission.)

this method is that the agarose filler has to be removed manually, limiting its applications in constructing more complex structures. For 4D printing, the aforementioned methods decreased operational difficulty and hold a potential to fabricate a relatively large vascular tissue construct. However, these methods only enable tubular structures with simple geometry (i.e., composed only one layer), whereas native vascular networks are commonly composed of three layers (i.e., vascular endothelial cells, vascular smooth muscle cells, and fibroblasts). To fabricate a complete vascular structure, more layers and cells should be further included.

15.4.2 Applications of 4D bioprinting in drug delivery

4D bioprinting provides a powerful tool to precisely control the spatial distribution of different components, which could self-fold or self-unfold. This can be employed to encapsulate and release cells or drugs in a programmable manner. For instance, multisomes in which networks of aqueous droplets with defined compositions are printed within small oil drops immersed in water ([Figure 15.7a](#)) [13]. The encapsulated droplets adhere to one another and to the surface of the oil drop and form interface bilayers, which allow communications between them and with the surrounding aqueous environment through membrane pores. By changing the temperature or pH value of the surrounding environment, the contents in the droplets can be released in a controllable manner. Such multicompartment framework of multisomes can work as a minisize bioreactor and has potential applications in delivery. The multisomes can also be functionalized with membrane proteins, for instance, to allow rapid electrical communication along a designed path. The multisome networks can also be programmed by osmolarity gradients to fold into different structures ([Figure 15.7b](#)), leading to the encapsulating and releasing of the desirable contents, which may find plenty of applications in the drug delivery [14]. Moreover, bioprinted bilayer structures that are self-foldable or self-unfoldable with respect to temperature have been successfully used for yeast cell encapsulation and release [22].

Another self-folding device enabling directional encapsulation and release of therapeutics was fabricated by two-layer hydrogels with different swelling ratios. This device is composed of two differentially swelling layers and a mucoadhesive drug layer that is attached on the bilayer. The bilayer structure includes a pH-sensitive hydrogel layer based on cross-linked poly(methacrylic acid) (PMAA) that swells significantly when in contact with body fluids, whereas the other is a nonswelling layer based on poly(hydroxyethyl methacrylate) (PHEMA). The self-folding device curls into the mucus due to the different swelling of the bilayered structure, leading to enhanced mucoadhesion. The nonswelling PHEMA layer can also serve as a diffusion barrier, minimizing any drug leakage in the intestine.

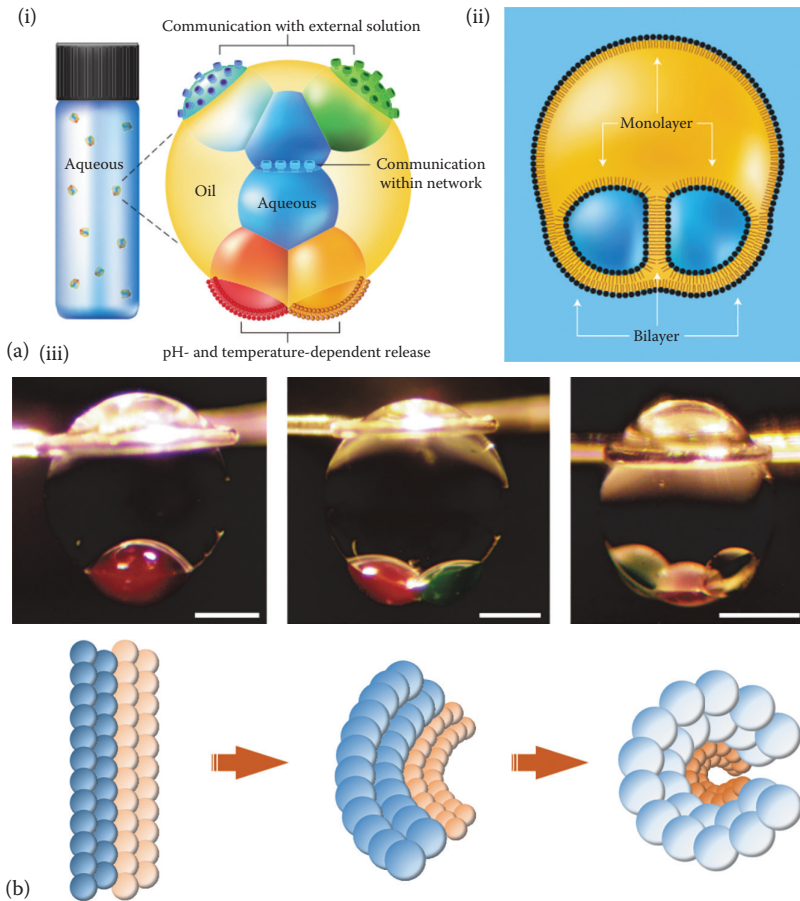


Figure 15.7 4D bioprinting based on droplet network, which could be used for drug delivery. (a) A schematic illustration of a bioprinted aqueous droplets network: (i) The aqueous droplets were encapsulated in an oil drop and were connected by lipid bilayers, which allow the droplets to communicate with the bulk solution. The droplet network can release the aqueous droplets by changing the pH value or temperature, (ii) schematic of a droplet network, which contains two droplets, and (iii) images of droplet network, which encapsulates one, two, and three droplets. Scale bars, 400 μm . (Reproduced from Villar, G. et al., *Nat. Nanotechnol.*, 6, 803–808, 2011. With permission.) (b) Droplet networks than enable self-folding. A droplet network, which contains two strips of droplets with different osmolarities. The droplet network would deform spontaneously due to the transfer of water.

Acid orange 8 and bovine serum albumin (BSA) were encapsulated in the bilayered structure and used for successful unidirectional release of drugs that transport through the mucosal epithelium [64].

15.5 Concluding remarks and future outlook

To sum up, the recently emerged technique of 4D bioprinting is considered as one of the most promising tools in biomedical engineering, in which the *time* as the fourth dimension incorporates with 3D bioprinting. In this chapter, we discussed two types of 4D bioprinting (i.e., the first one is based on self-deformation of *smart* materials and the second one is based on the development/maturation of cellular constructs) together with the potential applications. 4D bioprinting holds great potential to address the issues existing in 3D-bioprinting approach, such as the static and inanimate of the printed biomaterials and neglect of the dynamic change of the printed materials (e.g., scaffolds and cells), which are important for functional tissue regeneration. The 4D bioprinting holds a great promise in fabrication of engineered functional tissue constructs and controllable drug delivery. Although certain progress has been made so far, 4D bioprinting is still in its early stage and some challenges need to be addressed.

First, the responsive *smart* materials that can be printed are very limited and most of them are responsive to only one stimulus. However, in human body, the complicated microenvironment is stabilized by multiple regulations such as neuroregulation, self-regulation, and hormonal regulation. Thus, the 4D-bioprinted materials that enable the self-folding and are responsive to multiple physiologic signals are favorable for biomedical applications. In the future, we could 4D print the microcapsules that would self-deform due to the stimulus of gastric acid when arriving at the gastrophelcosis location to cover the gastrophelcosis wound and prevent further corrosion from the gastric acid. Besides, the released drugs could help curing the gastrophelcosis. Another advantage of 4D bioprinting is that it can customize for each patient, which could avoid the defect of pattern treatment and achieve the precision treatment. Besides discovering novel materials, making the existing responsive materials printable and biocompatible is another task in the future.

Second, the deformation patterns of the existing 4D-bioprinting methods are still limited to simple ones such as folding/unfolding or self-assembling. Aside from this, the deformation is still at macroscale, which restricts the precise manipulation of the structure of the printed construct. The future task is controlling the printed constructs at the microscale, changing their shapes, orientations inside the materials, or functionality. To increase the deformation accuracy, improving the printing resolution could be one solution. Another effort which should be put forth on is taking the spatially varying stimulus. For instance, using laser as the

heating source to deform the thermal responsive material may enable the precise deformation. In conclusion, 4D bioprinting is opening a new door in biomedical engineering and will serve as a toolbox to solve problems in TE and drug delivery.

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References

1. M. Gou, X. Qu, W. Zhu, M. Xiang, J. Yang, K. Zhang, Y. Wei, S. Chen, Bio-inspired detoxification using 3D-printed hydrogel nanocomposites, *Nature Communications*, 5 (2014) 3774.
2. J. Visser, F.P.W. Melchels, J.E. Jeon, E.M. van Bussel, L.S. Kimpton, H.M. Byrne, W.J.A. Dhert, P.D. Dalton, D.W. Huttmacher, J. Malda, Reinforcement of hydrogels using three-dimensionally printed microfibrils, *Nature Communications*, 6 (2015) 6933.
3. Q. Yang, Q. Lian, F. Xu, Perspective: Fabrication of integrated organ-on-a-chip via bioprinting, *Biomicrofluidics*, 11(3) (2017) 031301.
4. D. Kokkinis, M. Schaffner, A.R. Studart, Multimaterial magnetically assisted 3D printing of composite materials, *Nature Communications*, 6 (2015) 8643.
5. Z. Qin, B.G. Compton, J.A. Lewis, M.J. Buehler, Structural optimization of 3D-printed synthetic spider webs for high strength, *Nature Communications*, 6 (2015) 7038.
6. S.V. Murphy, A. Atala, 3D bioprinting of tissues and organs, *Nature Biotechnology*, 32(8) (2014) 773–785.
7. I. Ursan, L. Chiu, A. Pierce, Three-dimensional drug printing: A structured review, *Journal of the American Pharmacists Association*, 53(2) (2013) 136–144.
8. Q. Ge, C.K. Dunn, H.J. Qi, M.L. Dunn, Active origami by 4D printing, *Smart Materials and Structures*, 23(9) (2014) 094007.
9. S. Tibbits, 4D printing: Multi-material shape change, *Archit Design*, 84(1) (2014) 116–121.
10. W.G. Yang, H.B. Lu, W.M. Huang, H.J. Qi, X.L. Wu, K.Y. Sun, Advanced shape memory technology to reshape product design, manufacturing and recycling, *Polymers-Basel*, 6(8) (2014) 2287–2308.
11. M.M. Stanton, J. Samitier, S. Sanchez, Bioprinting of 3D hydrogels, *Lab on a Chip*, 15(15) (2015) 3111–3115.
12. K. Zhong Xun, J. Ee Mei Teoh, L. Yong, C. Chee Kai, Y. Shoufeng, A. Jia, L. Kah Fai, Y. Wai Yee, 3D printing of smart materials: A review on recent progresses in 4D printing, *Virtual and Physical Prototyping*, 10(3) (2015) 103–122.
13. G. Villar, A.J. Heron, H. Bayley, Formation of droplet networks that function in aqueous environments, *Nature Nanotechnology*, 6(12) (2011) 803–808.
14. G. Villar, A.D. Graham, H. Bayley, A tissue-like printed material, *Science*, 340(6128) (2013) 48–52.
15. N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Hydrogels in pharmaceutical formulations, *European Journal of Pharmaceutics and Biopharmaceutics*, 50(1) (2000) 27–46.

16. M. Jamal, S.S. Kadam, R. Xiao, F. Jivan, T.-M. Onn, R. Fernandes, T.D. Nguyen, D.H. Gracias, Bio-origami hydrogel scaffolds composed of photocrosslinked PEG bilayers, *Advanced Healthcare Materials*, 2(8) (2013) 1142–1150.
17. X. Wang, Y. Sun, C. Peng, H. Luo, R. Wang, D. Zhang, Transitional suspensions containing thermosensitive dispersant for three-dimensional printing, *ACS Applied Materials & Interfaces*, 7(47) (2015) 26131–26136.
18. S.E. Bakarich, R. Gorkin, M.I.H. Panhuis, G.M. Spinks, 4D printing with mechanically robust, thermally actuating hydrogels, *Macromolecular Rapid Communications*, 36(12) (2015) 1211–1217.
19. Y.M. Lee, S.H. Kim, C.S. Cho, Synthesis and swelling characteristics of pH and thermo-responsive interpenetrating polymer network hydrogel composed of poly(vinyl alcohol) and poly(acrylic acid), *Journal of Applied Polymer Science*, 62(2) (1996) 301–311.
20. Q. Ge, H.J. Qi, M.L. Dunn, Active materials by four-dimension printing, *Applied Physics Letters*, 103(13) (2013) 131901.
21. J.C. Breger, C. Yoon, R. Xiao, H.R. Kwag, M.O. Wang, J.P. Fisher, T.D. Nguyen, D.H. Gracias, Self-folding thermo-magnetically responsive soft microgrippers, *ACS Applied Materials & Interfaces*, 7(5) (2015) 3398–3405.
22. G. Stoychev, N. Pureskiy, L. Ionov, Self-folding all-polymer thermoresponsive microcapsules, *Soft Matter*, 7(7) (2011) 3277–3279.
23. S. Wang, W.Y. Yeong, J.M. Lee, Smart hydrogels for 3D bioprinting, *International Journal of Bioprinting*, 1(1) (2015) 14.
24. D.B. Kolesky, R.L. Truby, A. Gladman, T.A. Busbee, K.A. Homan, J.A. Lewis, 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs, *Advanced Materials*, 26(19) (2014) 3124–3130.
25. R. Censi, W. Schuurman, J. Malda, G. Di Dato, P.E. Burgisser, W.J. Dhert, C.F. Van Nostrum, P. Di Martino, T. Vermonden, W.E. Hennink, A printable photopolymerizable thermosensitive p (HPMAM-lactate)-PEG hydrogel for tissue engineering, *Advanced Functional Materials*, 21(10) (2011) 1833–1842.
26. J. Escobar-Chávez, M. López-Cervantes, A. Naik, Y. Kalia, D. Quintanar-Guerrero, A. Ganem-Quintanar, Applications of thermo-reversible pluronic F-127 gels in pharmaceutical formulations, *Journal of Pharmacy & Pharmaceutical Sciences*, 9(3) (2006) 339–358.
27. W. Schuurman, P.A. Levett, M.W. Pot, P.R. van Weeren, W.J. Dhert, D.W. Hutmacher, F.P. Melchels, T.J. Klein, J. Malda, Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs, *Macromolecular Bioscience*, 13(5) (2013) 551–561.
28. Y. Qiu, K. Park, Environment-sensitive hydrogels for drug delivery, *Advanced Drug Delivery Reviews*, 53(3) (2001) 321–339.
29. T. Tanaka, D. Fillmore, S.T. Sun, I. Nishio, G. Swislow, A. Shah, Phase-transitions in ionic gels, *Physical Review Letters*, 45(20) (1980) 1636–1639.
30. S.H. Yuk, S.H. Cho, S.H. Lee, pH/temperature-responsive polymer composed of poly((N, N-dimethylamino)ethyl methacrylate-co-ethylacrylamide), *Macromolecules*, 30(22) (1997) 6856–6859.
31. H.Y. Jiang, S. Kelch, A. Lendlein, Polymers move in response to light, *Advanced Materials*, 18(11) (2006) 1471–1475.
32. M.H. Li, P. Keller, B. Li, X.G. Wang, M. Brunet, Light-driven side-on nematic elastomer actuators, *Advanced Materials*, 15(7–8) (2003) 569–572.
33. J. Ryu, M. D'Amato, X.D. Cui, K.N. Long, H.J. Qi, M.L. Dunn, Photo-origami-Bending and folding polymers with light, *Applied Physics Letters*, 100(16) (2012) 161908.
34. Y. Lee, H. Lee, T. Hwang, J.G. Lee, M. Cho, Sequential folding using light-activated polystyrene sheet, *Scientific Reports*, 5 (2015) 16544.

35. C. Py, P. Reverdy, L. Doppler, J. Bico, B. Roman, C.N. Baroud, Capillary origami: Spontaneous wrapping of a droplet with an elastic sheet, *Physical Review Letters*, 98(15) (2007) 156103.
36. A. Antkowiak, B. Audoly, C. Josserand, S. Neukirch, M. Rivetti, Instant fabrication and selection of folded structures using drop impact, *Proceedings of the National Academy Sciences USA*, 108(26) (2011) 10400–10404.
37. C. Py, P. Reverdy, L. Doppler, J. Bico, B. Roman, C. Baroud, Capillarity induced folding of elastic sheets, *The European Physical Journal Special Topics*, 166(1) (2009) 67–71.
38. K. Kuribayashi-Shigetomi, H. Onoe, S. Takeuchi, Cell origami: Self-folding of three-dimensional cell-laden microstructures driven by cell traction force, *PLoS One*, 7(12) (2012) e51085.
39. H. Ding, W. Liu, Y. Ding, J. Shao, L. Zhang, P. Liu, H. Liu, Particle clustering during pearl chain formation in a conductive-island based dielectrophoretic assembly system, *Rsc Advances*, 5(8) (2015) 5523–5532.
40. H. Ding, W. Liu, J. Shao, Y. Ding, L. Zhang, J. Niu, Influence of induced-charge electrokinetic phenomena on the dielectrophoretic assembly of gold nanoparticles in a conductive-island-based microelectrode system, *Langmuir*, 29(39) (2013) 12093–12103.
41. H. Ding, J. Shao, Y. Ding, W. Liu, H. Tian, X. Li, One-dimensional Au–ZnO hetero-nanostructures for ultraviolet light detectors by a two-step dielectrophoretic assembly method, *ACS Applied Materials Interfaces*, 7(23) (2015) 12713–12718.
42. H. Ding, J. Shao, Y. Ding, W. Liu, X. Li, H. Tian, Y. Zhou, Effect of island shape on dielectrophoretic assembly of metal nanoparticle chains in a conductive-island-based microelectrode system, *Applied Surface Science*, 330 (2015) 178–184.
43. W. Liu, C. Wang, H. Ding, J. Shao, Y. Ding, AC electric field induced dielectrophoretic assembly behavior of gold nanoparticles in a wide frequency range, *Applied Surface Science*, 370 (2016) 184–192.
44. Q. Zhao, W. Wang, J. Shao, X. Li, H. Tian, L. Liu, X. Mei, Y. Ding, B. Lu, Nanoscale electrodes for flexible electronics by swelling controlled cracking, *Advanced Materials*, 28(30) (2016) 6337–6344.
45. D. Kokkinis, M. Schaffner, A.R. Studart, Multimaterial magnetically assisted 3D printing of composite materials, *Nature Communications*, 6 (2015) 8643.
46. Z.X. Khoo, J.E.M. Teoh, Y. Liu, C.K. Chua, S. Yang, J. An, K.F. Leong, W.Y. Yeong, 3D printing of smart materials: A review on recent progresses in 4D printing, *Virtual and Physical Prototyping*, 10(3) (2015) 103–122.
47. V.K. Lee, D.Y. Kim, H. Ngo, Y. Lee, L. Seo, S.-S. Yoo, P.A. Vincent, G. Dai, Creating perfused functional vascular channels using 3D bio-printing technology, *Biomaterials*, 35(28) (2014) 8092–8102.
48. N.E. Fedorovich, J. Alblas, W.E. Hennink, F.C. Öner, W.J.A. Dhert, Organ printing: The future of bone regeneration? *Trends in Biotechnology*, 29(12) (2011) 601–606.
49. J.S. Miller, K.R. Stevens, M.T. Yang, B.M. Baker, D.-H.T. Nguyen, D.M. Cohen, E. Toro et al., Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues, *Nature Materials*, 11(9) (2012) 768–774.
50. D.B. Kolesky, R.L. Truby, A.S. Gladman, T.A. Busbee, K.A. Homan, J.A. Lewis, 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs, *Advanced Materials*, 26(19) (2014) 3124–3130.
51. A.M. Blakely, K.L. Manning, A. Tripathi, J.R. Morgan, Bio-pick, place, and perfuse: A new instrument for three-dimensional tissue engineering, *Tissue Engineering Part C-Methods*, 21(7) (2015) 737–746.
52. V. Mironov, R.P. Visconti, V. Kasyanov, G. Forgacs, C.J. Drake, R.R. Markwald, Organ printing: Tissue spheroids as building blocks, *Biomaterials*, 30(12) (2009) 2164–2174.

53. A.L. Rutz, K.E. Hyland, A.E. Jakus, W.R. Burghardt, R.N. Shah, A multimaterial bioink method for 3D printing tunable, cell-compatible hydrogels, *Advanced Materials*, 27(9) (2015) 1607–1614.
54. Y. Li, G. Huang, X. Zhang, L. Wang, Y. Du, T.J. Lu, F. Xu, Engineering cell alignment in vitro, *Biotechnology Advances*, 32(2) (2014) 347–365.
55. Y. Li, C.T. Poon, M. Li, T.J. Lu, B. Pingguan-Murphy, F. Xu, Chinese-noodle-inspired muscle myofiber fabrication, *Advanced Functional Materials*, 25(37) (2015) 5999–6008.
56. Y. Li, G. Huang, B. Gao, M. Li, G.M. Genin, T.J. Lu, F. Xu, Magnetically actuated cell-laden microscale hydrogels for probing strain-induced cell responses in three dimensions, *NPG Asia Materials*, 8(1) (2016) e238.
57. J.U. Lind, T.A. Busbee, A.D. Valentine, F.S. Pasqualini, H. Yuan, M. Yadid, S.-J. Park, A. Kotikian, A.P. Nesmith, P.H. Campbell, Instrumented cardiac microphysiological devices via multimaterial three-dimensional printing, *Nature Materials*, 16 (2016) 303–308.
58. R.K. Jain, P. Au, J. Tam, D.G. Duda, D. Fukumura, Engineering vascularized tissue, *Nature Biotechnology*, 23(7) (2005) 821–823.
59. J. Malda, T.J. Klein, Z. Upton, The roles of hypoxia in the in vitro engineering of tissues, *Tissue Engineering*, 13(9) (2007) 2153–2162.
60. C. Norotte, F.S. Marga, L.E. Niklason, G. Forgacs, Scaffold-free vascular tissue engineering using bioprinting, *Biomaterials*, 30(30) (2009) 5910–5917.
61. S. Hong, S.-J. Song, J.Y. Lee, H. Jang, J. Choi, K. Sun, Y. Park, Cellular behavior in micropatterned hydrogels by bioprinting system depended on the cell types and cellular interaction, *Journal of Bioscience and Bioengineering*, 116(2) (2013) 224–230.
62. G.Y. Huang, L.H. Zhou, Q.C. Zhang, Y.M. Chen, W. Sun, F. Xu, T.J. Lu, Microfluidic hydrogels for tissue engineering, *Biofabrication*, 3(1) (2011) 012001.
63. Y.N. Yan, X.H. Wang, Y.Q. Pan, H.X. Liu, J. Cheng, Z. Xiong, F. Lin, R.D. Wu, R.J. Zhang, Q.P. Lu, Fabrication of viable tissue-engineered constructs with 3D cell-assembly technique, *Biomaterials*, 26(29) (2005) 5864–5871.
64. H.Y. He, J.J. Guan, J.L. Lee, An oral delivery device based on self-folding hydrogels, *Journal of Controlled Release*, 110(2) (2006) 339–346.

Chapter 16 Current challenges and future perspectives of bioprinting

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Tissue Engineering (TE) aims to build biological substitutes that mimic native tissues that can be used to replace damaged tissues or restore compromised organs (Atala 2009). Over the past three decades, TE has evolved into a multidisciplinary field involving biologists, chemists, material scientists, and surgeons (Dvir et al. 2011). It involves the directed application of biomaterials, cells, and biologically active factors to affect the tissue formation. TE of organs starts with taking a small biopsy from the patient, isolating cells from the biopsy, expanding the cells *in vitro* and seeding them on natural scaffolds such as collagen, fibrin, and alginate or synthetic biomaterial scaffolds such as polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), and so on (Atala 2009). The cells proliferate and differentiate in the scaffold, and the resulting tissue construct is allowed to mature in a bioreactor for a period of couple of days to couple of weeks depending on the tissue or organ of interest (Murphy and Atala 2013). The mature tissue construct is then used for transplantation or *in vitro* testing. Over the past decade, there have been quite a few successes in terms of engineered tissues. Different tissues and organs have been fabricated, including skin and

cornea (Bottcher-Haberzeth et al. 2010; Fagerholm et al. 2010), which have a flat structure, blood vessel grafts, urethra and trachea, which are tubular (Shin'oka et al. 2001; L'Heureux et al. 2007; Macchiarini et al. 2008; Dahl et al. 2011; Raya-Rivera et al. 2011), and bladder and vagina (Atala et al. 2006; Raya-Rivera et al. 2014), which are hollow organs. Currently, there are ongoing efforts from several research groups to engineer more complex solid organs such as kidney, liver, and heart.

3D printing is adding a new dimension to TE. 3D printing can automate TE and facilitate cost-effective large-scale manufacturing. 3D printing, originally called *stereolithography (SL)* is an additive manufacturing technique, which was first developed by Charles W. Hull in 1986 (Murphy and Atala 2014). It involves sequential printing of thin layers of a material and curing it with ultraviolet light to form a solid 3D structure. 3D bioprinting is a variant of 3D printing, it is a computer-aided manufacturing process that deposits living cells together with hydrogel-based scaffolds and allows for patterning of individual components of the tissue or organ, thereby facilitating formation of complex tissue architecture (Mironov et al. 2009). 3D bioprinting for fabricating biological constructs typically involves layer-by-layer addition of material on a supporting scaffold to build 3D tissue or organ constructs with input from a computer-aided design (CAD) file (Guillemot et al. 2010b). Using bioprinting tissue or organ constructs can be tailor-fabricated by suitably altering the CAD file prior to printing (Marga et al. 2012). 3D-bioprinting technology involves six different steps, briefly, imaging of the target tissue or organ is done using either magnetic resonance or computed tomography imaging, then using the imaging input the model is developed with CAD–CAM (computer-aided manufacture) software, depending on the tissue or organ to be printed the biomaterial scaffolds and cells are carefully chosen, one or more cell types could be used (Murphy and Atala 2014). The tissue is printed using a bioprinter and allowed to mature in a bioreactor ranging from a few days to a couple of weeks. Subsequently, depending on the intended application the tissue constructs are used either for implantation or *in vitro* testing (Visscher et al. 2016). The main technological approaches for deposition of cells include inkjet-, microextrusion-, and laser-based bioprinting (Murphy and Atala 2014). Inkjet-based bioprinting utilizes thermal-, piezo-, or acoustic-driven mechanisms to deposit droplets of cell suspension in a high-throughput manner (Murphy and Atala 2014). Although there are many advantages to inkjet bioprinting technology, a downside is the risk of exposing cells and materials to thermal and mechanical stress, and in the case of acoustic printers, to frequencies that may damage the cell membrane. Inkjet bioprinters are also limited by the viscosity of the bioink because the more viscous the bioink the greater the force required to eject the droplet from the printer nozzle (Murphy and Atala 2014). In addition, to prevent clogging of the nozzle of the inkjet bioprinter the cell density that could be used is significantly lower than physiologically relevant numbers. Microextrusion

bioprinting uses mechanical or pneumatic dispensing systems to extrude continuous beads of material, often consisting of cells mixed with hydrogels (Murphy and Atala 2014). Structures are printed in 2D with hydrogel and the material is then solidified either physically or chemically so that the structures could be combined to create 3D shapes. Microextrusion printers allow for a wider selection of biomaterials since more viscous materials could be printed. Another advantage is that the printer can deposit very high cell densities. While cell viability is lower than that obtained with inkjet printers, viability is in the range of 40%–86%, depending on the nozzle gauge and extrusion pressure. A third printing approach, laser-assisted bioprinting, is based on the principles of laser-induced forward transfer (LIFT). This printing system involves the use of a pulsed laser beam, a focusing system, and a *ribbon* that has a donor transport support, a layer of biological material, and a receiving substrate facing the ribbon (Guillemot 2010a; Koch et al. 2013). Focused laser pulses are used to generate a high-pressure bubble that propels cell-containing materials toward the collector substrate. Because the technology is nozzle free, there is no problem with cell clogging. Other advantages of laser-assisted bioprinting include printer compatibility with a range of bioink viscosities; it can deposit very high density and can print mammalian cells without affecting the cell viability (Koch et al. 2010; Gruene et al. 2011a; Hribar et al. 2014). The main disadvantage of this is that the high resolution results in a low overall flow rate, which can make it difficult to accurately target and position cells. Also there is a possibility that metallic residues may be present in the final construct (Guillotin and Guillemot 2011; Ferris et al. 2013). [Table 16.1](#) shows a comparison of the different bioprinting techniques. The recent development of the integrated tissue-organ printer (ITOP) by our group allows for bioprinting of human-scale tissues of any shape (Kang et al. 2016). With earlier bioprinting strategies, bioprinting of complex and stable human-scale tissues was not possible due to the lack of structural stability of larger printed structures and the lack of sufficient oxygen and nutritional support beyond the 200 μm diffusion limit in tissues. The ITOP facilitates bioprinting with very high precision; it has a resolution of 50 μm for cells and 2 μm for scaffolding materials. [Figure 16.1](#) shows the ITOP system and the process followed for automated 3D bioprinting. This enables recapitulation of heterocellular tissue biology and allows for fabrication of functional tissues. The ITOP is configured to

Table 16.1 Comparison of the Different Bioprinting Techniques

	Inkjet Bioprinting	Microextrusion Bioprinting	Laser-Assisted Bioprinting
Printing process	Drop-by-drop	Line-by-line	Dot-by-dot
Printing speed	Medium (mm/s)	Slow (10–50 $\mu\text{m/s}$)	Medium (mm/s)
Resolution	50 μm	5 μm	<500 nm
Cell viability	>85%	40%–80%	>85%

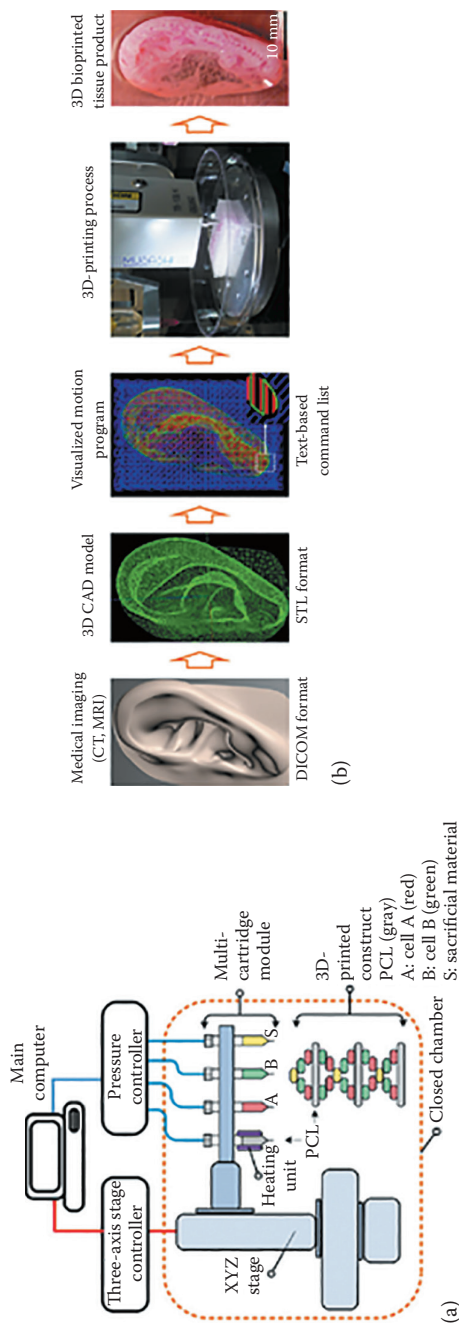


Figure 16.1 The ITOP system (a) the three-axis stage, dispensing module, and temperature-controlled closed chamber, (b) the bioprinting process followed: Medical imaging, model development, motion program generation, and printing. (Modified from Kang, H.W. et al., *Nat. Biotechnol.*, 34, 312–319, 2016. With permission.)

deliver the bioink within a stronger water-soluble gel, Pluronic F-127, that helps the printed cells to maintain their shape during the printing process. Thereafter, the Pluronic F-127 scaffolding could be simply washed away from the bioprinted tissue. To ensure adequate oxygen diffusion into the bioprinted tissue, microchannels can be created with the biodegradable polymer, PCL. Stable human-scale ear cartilage, bone and skeletal muscle structures were printed with the ITOP, which when implanted in animal models, matured into functional tissue and developed a network of blood vessels and nerves.

16.1 Challenges and future directions

3D bioprinting has added a new dimension to TE and regenerative medicine and has great potential to transform the field. Bioprinting for TE and regenerative medicine includes the design, prototyping, and fabrication of three-dimensional (3D) tissue structures (e.g., skin, cartilage, and bone) that could be used for therapies. Bioprinting could be used to either develop mass use *off-the-shelf* products that would be used by many patients or custom products that would be used by specific end users. In the latter case, developing a 3D bioprinter that could be housed in a patient facility or hospital setting would enable real-time biofabrication of a product that could be brought into the surgical suite. In the case of *off-the-shelf* products, issues related to quality control, release testing, packaging, shipping, and logistics would need to be determined and established for each product. Although much progress has been made in 3D bioprinting, there is a need for standardization and integration of an entire biofabrication platform, beginning with software design for building models to post-processing of the printed tissues. To enable widespread adoption of the technology there are several challenges that need to be overcome in the pre-printing, printing, and post-printing tissue maturation stages of bioprinting.

16.2 Tissue pre-printing stage

This stage of bioprinting involves isolation of cells from the tissue biopsy, expansion of the cells, differentiation of the cells, and preparation of the bioink, which is made of cells and biomaterial support materials. The cells could be patient- and organ-specific primary or stem cells. Primary cells are fully differentiated cells that could be specifically isolated from the tissue of interest and expanded *in vitro* and used. On the other hand, stem cells need to be differentiated into the tissue of interest before use in printing. The choice of cells would depend on the application. In the case of healthy tissues, primary cells could be isolated, expanded, and used; however, in the case of diseased tissue, stem cells may have to be differentiated into the cells of interest and used. Stem cells could be from different sources: adipose, mesenchymal, perinatal, and induced pluripotent stem

cells. Also, one type of cell or multiple types of cells could be used for printing. Very huge numbers of cells are required for printing of organs; billions of cells would be needed to bioprint organs with physiologically equivalent cell numbers. Currently, the cell expansion technologies we have could easily expand cells in millions, innovative cell expansion technologies need to be developed to bioprint organs for transplantation. Also, more effective reagents for maintaining cells in their state of differentiation will be required. Further, development of animal-factor free reagents will facilitate easier clinical translation of these technologies. This platform must include superior bioinks that allow for complex, reproducible design of 3D tissues.

16.2.1 Bioinks for biofabrication

Bioinks form the delivery medium that encapsulates the cells and minimize cell injury during the printing process, providing a supportive microenvironment for post-printing maturation of the microtissue. The choice of bioink is a critical aspect of bioprinting that is essential for the cells to be deposited in specific patterns on the basis of the CAD models and is chosen on the basis of the physiology and function of the tissue to be printed. The function of connective tissue such as bone and cartilage depends to a great extent on the tissue mechanical properties, whereas organs such as liver and lungs rely on their intricate vascular networks. Attempts to mimic the diverse tissue architecture and composition of the various tissue and organ types have led to development of a variety of bioinks for 3D bioprinting. Appropriate choice of bioink is essential for providing the chemical and physical cues to facilitate necessary cell–ECM interactions; bioink properties not only affect the cell growth, proliferation, and differentiation but also the structure and function of fabricated tissues. It is essential that the bioinks be biocompatible and cell supportive, they should facilitate functional differentiation of the cells into the target tissues. Other important bioink properties to consider include gelation kinetics, rheological characteristics, cell–matrix interactions, material properties, and cell source. Typically, the bioinks could physically serve as cell-laden hydrogels or sacrificial support materials that are removed immediately after printing or as mechanical support materials that provide specific mechanical characteristics to the tissue. Bioinks can be fully natural materials such as collagen, fibrin, hyaluronic acid (HA), and alginate, which could be used in the form of hydrogels for the cells, or synthetic materials such as polycaprolactone (PCL), polylactide (PLA), polyglycolide (PGA), poly(lactic-co-glycolic acid) (PLGA), and polyethylene glycol (PEG) polymers or hybrid biomaterials that contain a combination of natural and synthetic materials, which could provide mechanical support (Arslan-Yildiz et al. 2016). On the other hand, materials such as Pluronic-F127 could be used as sacrificial support materials that keep the cells together while printing and could be simply washed away following the printing of the tissue construct (Kang et al. 2016).

To achieve a desired tissue construct, it is essential to control the rheological properties of bioprinted hydrogel matrix and matrix stiffness that will result in optimal tissue maturation and development. Printability of the bioink indicates the ease with which it could be printed with good resolution and its ability to maintain its structure for post-printing tissue maturation. The bioink formulation should be stable enough so as to provide architectural stability to the tissue construct. Shape fidelity and printing resolution are other important considerations when assessing the printability of the bioink (Kirchmayer 2015). Ideally, the viscosity of the bioink should be such that it is not only supportive for cell growth and differentiation but also suitable for printing, but in reality, viscosities appropriate for bioprinting may not be supportive of cell viability. So to achieve good printability and at the same time to ensure high cell viability, the printing conditions and bioink consistency need to be optimized. Bioinks could either be Newtonian or non-Newtonian in nature; both kinds have been optimized for better printability. In the case of non-Newtonian bioinks, printable viscosity is determined as the extent of strain that occurs during bioprinting, which depends on the concentration of the bioink and its molecular weight. For these kinds of bioinks, their shear thinning nature could be enhanced to increase the cell viability and printing resolution (Highley et al. 2015). Shear thinning of the bioink refers to the decrease in its viscosity with increase in rates of strain (Panwar and Tan 2016). This is due to the fact that shear thinning decreases the shear stress experienced by cells and allows for smooth extrusion of the viscous bioink through the nozzle resulting in higher cell viability. The high viscosity of the bioink ensures that the extruded filament retains its shape and as a result maintains print fidelity. Other factors such as nozzle shape, size, and temperature also contribute to bioink extrusion rate and consistency, which in turn affect the printability, structural integrity, resolution, and shear stress experienced by cells in the bioink (Panwar and Tan 2016). For example, bioinks with low viscosities and low thermal conductivity are typically preferred for inkjet bioprinting as they prevent clogging of the printing nozzles and heat-induced loss of cell viability. Although shear thinning bioinks with higher viscosities are preferred for extrusion bioprinting, a shear-thinning material is a non-Newtonian material that decreases in viscosity with a corresponding increase in the shear rate (Panwar and Tan 2016). Material properties such as hydration, degradation rate, mechanical properties, and permeability are also very important for bioinks. Permeability and hydration can affect viscosity of a bioink as well as oxygen and nutrient transport to cells, mechanical strength of a final construct, and swelling properties (Chung 2013). Degradation rate and mechanical properties can affect cell growth and differentiation as well as long-term biocompatibility (Chung 2013).

Additional biomaterials that could be used for development of bioinks will need to be identified, due to the diverse nature of tissues being bioprinted, ranging from skin to liver to bone. Bioink development should be done with a focus on the

different biomechanical and structural requirements of the different tissue types. As we advance in our ability to bioprint individual tissues and organs, and potentially attempt to bioprint composite tissue that may contain a mix of soft and hard tissue such as skin, skeletal muscle, and bone, we will need to develop some sort of standard or universal bioink that could support different tissue types without compromising functionality. Another important factor that should be considered is how quickly the material will degrade in the body; the cells should be able to degrade the scaffold at a rate that would match their extracellular production and remodeling activity.

16.3 Bioprinting stage

To initiate bioprinting the print files that contain accurate surface information of complex 3D geometries are converted to the Stereolithography file format with coordinates for the print head path (Mironov et al. 2009; Mondy et al. 2009). These files contain accurate surface information required to reconstruct the complex 3D model and can be designed using CAD–CAM graphic user interfaces or created from clinical images with input from magnetic resonance imaging (MRI) and computed tomography (CT) imaging (Keriquel et al. 2010; Arai et al. 2011). The paths for the print heads are created by slicing the STL model into layers and creating bioprinter toolpaths that trace out the perimeter and interior features of each slice. The thickness of each of these slices is referred to as the resolution of the particular printer and is usually in the 100–500 μm range. Resolution is specific to the printer used and also depends on the type of bioink used for printing; the smaller the resolution the better the quality but longer the print time. The bioprinter reads the SL files and layer-by-layer deposits the bioink to build the 3D tissue or organ from the series of 2D slices. High-quality image acquisition is essential for high-fidelity bioprinting of the tissue of interest. The printing resolution and viability of cells post-printing would significantly vary depending on the bioprinting modality employed. Clinical images can provide information regarding the *in vivo* cell distribution in the tissue or organs of patients and image processing tools can be used to determine anatomically realistic tissue geometries. With increasing complexity of the organs; for printing solid organs such as liver, kidney, and heart; and also for composite tissue that contain different tissue types, the printing resolution will need to be improved to duplicate their intricate inner microarchitecture. The ability to print microscale features is necessary for cellular function. Better control over the microarchitecture will enable fabricated tissue to recapitulate the native form and function of the tissue of interest. Increasing the printing speed is another challenge; current approaches that facilitate higher printing speed such as extrusion bioprinting can compromise the integrity of cells and cause significant loss in their viability. CAD–CAM can also be used to predict

the feasibility of the fabrication process by simulating relevant physical models using both classical formula calculations and finite element methods (FEM). Currently, the most widely used physical model for bioprinting is laminar multiphase flow; although it is an oversimplified model and ignores issues related to inclusion of cells, the simulations are useful for checking and optimizing the feasibility of specific designs.

Building a functional vasculature is one of the most fundamental challenges in TE; the ability to 3D-bioprint vasculature will enable fabrication of a pre-formed microvascular network that can better anastomose to the host circulation and achieve functional perfusion within the tissue-engineered construct (Laschke et al. 2009; Auger et al. 2013). The use of sacrificial inks to create 3D-interconnecting networks, which can be removed after printing the entire construct, leaving hollow channels for the perfusion of endothelial cells and formation of blood vessel network is a promising approach. Miller et al. (2012) have shown how 3D-extrusion printing and cast molding could be combined to create a 3D-interconnected perfusable vasculature. However, this molding technique is limited to the construction of simple block tissue architectures. Recently, a bioprinting approach that enables the simultaneous printing of the vasculature structure and the surrounding cells for heterogeneous cell-laden tissue constructs has been reported by the research group of Jennifer Lewis. They have developed a method that involves the use of Pluronic F127 as a fugitive bioink, which can be printed and dissolved under mild conditions, enabling printing of heterogeneous cell-laden tissue constructs with interconnecting vasculature networks (Kolesky et al. 2014). There have also been attempts to bioprint the vascular network directly; Zhang et al. recently reported about direct bioprinting of vessel-like cellular microfluidic channels with hydrogels, such as alginate and chitosan using a coaxial nozzle (Yu et al. 2013). In very recently reported work from the Jennifer Lewis lab, they have demonstrated bioprinting of 3D cell-laden, vascularized tissues that exceed 1 cm in thickness and can be perfused on chip for greater than six weeks (Kolesky et al. 2016). They integrated parenchyma, stroma, and endothelium into a single thick tissue by co-printing multiple inks composed of human mesenchymal stem cells and human neonatal dermal fibroblasts within a customized fibrin–gelatin matrix alongside embedded vasculature, which was subsequently lined with human umbilical vein endothelial cells. This may open up newer avenues for printing of prevascularized tissues. To print vascularized tissue models with complexity and resolution matching *in vivo* structures, we need to improve print resolution and reduce the printing time. The ability to bioprint hierarchical vascular networks while building complex tissue will be critical to fabrication of transplantable organs. Also, critical for production of vascularized tissues using 3D bioprinting will be the ability to recapitulate vascular flow *in vitro* (Paulsen and Miller 2015).

16.4 Post-printing organ or tissue maturation stage

Immediately following printing, the tissue constructs are fragile; they need to be matured in a bioreactor for a period of couple of days to couple of weeks before they are used for transplantation. The tissue maturation stage will ensure that the cells have proliferated in the construct and differentiated to form functional tissue components. Different cell types have different nutritional and metabolic requirements, so different kinds of cells require different kinds of growth media. Development of a standard or universal growth media for cells would be beneficial for growth and maturation of composite tissue constructs prior to transplantation. The cells also are in dynamic reciprocity with their microenvironment, which includes the extracellular matrix (ECM) in which they are embedded in. The cells secrete proteins, proteases, and other metabolites onto the ECM, which facilitate dynamic homeostatic phase of tissue remodeling. Inclusion of native ECM in the bioink will ensure the presence of natural ligands for the cells, which will facilitate a suitable growth environment for the cells. Also, development of novel bioreactors to facilitate dynamic culture would facilitate physiologic-like environment for maturation of tissues that incorporate printed vasculatures. As bioprinting of more complex solid organs becomes possible, better analytical and computational approaches to effectively study the development and maturation of these tissues prior to transplantation will need to be developed. There has been a lot of effort to model bioprinted tissue with the corresponding printing parameters. For extrusion printing, relationships between dispensing pressure, printing time, and nozzle diameter have been tested and modeled (Yu et al. 2013). In inkjet printers, cell settling that occurs during printing and is known to cause clogging of the nozzles has been modeled by both analytical and finite element methods (FEM) (Mezel et al. 2010; Pepper et al. 2011, 2012). For laser printing, the effects of laser energy, substrate film thickness, and hydrogel viscosity on cell viability (Catros et al. 2011) as well as droplet size (Mezel et al. 2010, Gruene et al. 2011a), cell differentiation (Gruene et al. 2011b), and cell proliferation (Gruene et al. 2011b) have been studied. Researchers have also done post-printing modeling of cellular dynamics (McCune et al. 2014, de Leon et al. 2014), fusion (de Leon et al. 2014), deformation, and stiffness (Tirella et al. 2011).

16.5 Regulatory requirements on customized work processes

The clinical translation of tissue-engineered products is in its early phases; realization of efficient and cost-effective advanced manufacturing techniques would facilitate clinical translation of a range of tissue-engineered and bioprinted organs and tissues. Manufacturing challenges for bioprinted tissues and organ constructs are significant as they involve cells' active monitoring of cellular yields,

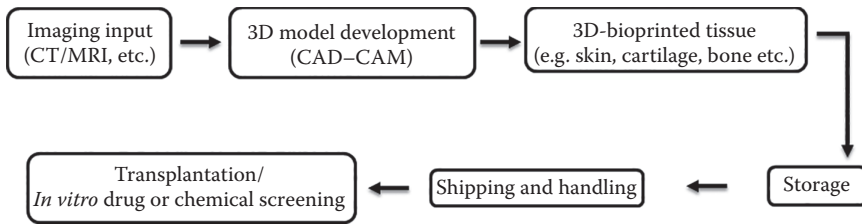


Figure 16.2 Typical manufacturing workflow for bioprinted tissues. (Modified from Hunsberger, J. et al., *Stem Cells Transl. Med.*, 4, 130–135, 2015. With permission.)

and maintenance of quality parameters such as purity, potency, and viability during production is critical. Also, as the bioinks contain ECM scaffold components, the quality of the scaffolds and potential for causing contamination and disease transmission will need to be checked along with real-time monitoring, and noninvasive release testing procedures that will need to be established before delivery of the bioprinted tissues to the patient (Hunsberger et al. 2015). [Figure 16.2](#) shows the manufacturing work flow for commercial-scale manufacturing of bioprinted tissue. To successfully translate organ bioprinting to the clinic, robust automated protocols and procedures will need to be established.

To ensure effective clinical translation and commercialization of 3D-bioprinting technology regulatory standards for quality assurance of bioinks, bioprinters, and bioprinted products are essential. A comprehensive regulatory framework to assure quality control during every step of the process—design of the model, selection of bioinks, bioprinting process, validation of the printing, post-printing maturation, and product quality assessment before transplantation is essential. The FDA recently issued the draft version on “Technical Considerations for Additive Manufactured Devices” to provide guidance on 3D-printing techniques for production of medical devices. Since bioprinted tissues are tissue-engineered organs, all criteria applicable to engineered tissue would apply to bioprinted tissue also. Tissue-engineered organs are typically combination products and may include pharmaceuticals, medical devices, biologics, and their use involves the application of surgical procedures. New surgical procedures are not regulated by the FDA and can be used on an *as needed* basis at the discretion of the surgeon performing the operation; these organ transplant procedures are regulated by Health and Human Services and not the FDA. But surgically implanted engineered organs or tissues, depending on their composition, fall under the purview of the FDA either as devices or biologics. New medical devices, on the other hand, are federally regulated; they are classified and regulated as either devices or biologics and need to be tested in clinical trials before a surgeon is allowed to use it initially. Currently, products that use stem cells or are derived from stem cells are treated by the FDA as somatic cellular therapies and are regulated as

biologics under Section 351 of the Public Health Act (Varkey and Atala 2015). As cellular therapies, they are also subject to FDA guidelines for the manufacture of human cells, tissues, and cellular and tissue-based products found in part 1271 of the same act. Part 1271 establishes the requirements for donor eligibility procedures not found in the current good manufacturing practices guidelines of parts 210 and 211 (Varkey and Atala 2015). These guidelines regulate the way stem cells are isolated, handled, and labeled. Also, engineered tissues typically used in research do not require FDA approval during animal and *in vitro* testing, if they are not intended for use on humans. However, Title 21 of the Federal Code of Regulations defines certain restrictions with regard to shipping and disposal of these products.

16.6 Conclusion

Bioprinting is a very advanced fabrication technique that could be used for rapid prototyping of cell-laden hydrogels to develop 3D tissue constructs. This technique offers unique advantages compared to conventional microfabrication methods, including the ability to precisely dispense bioink with high spatial and temporal resolution and build microscale tissue structures, and high-throughput manufacturing. Further improvements in all stages of application of the technology, including mechanical and rheological shortcomings of bioinks, printing speed and accuracy, ability to print vascular networks, and further development of novel dynamic post-printing tissue maturation systems will facilitate more widespread adoption and use of this technology in the clinic. Advances in bioprinting technology will not only help in development of bioprinted tissues and organs, it would contribute to development of novel disease models, and drug and chemical screening systems.

Abbreviations

CAD	Computer-aided design
CAM	Computer-aided manufacture
CT	Computed tomography
ECM	Extracellular matrix
FDA	Food and Drug Administration
FEM	Finite element methods
HA	Hyaluronic acid
ITOP	Integrated tissue and organ printer
LIFT	Laser-induced forward transfer
MRI	Magnetic resonance imaging
PCL	Polycaprolactone

PEG Polyethylene glycol
PGA Polyglycolic acid
PLA Polylactic acid
PLGA Poly(lactic-co-glycolic) acid
SL Stereolithography

References

- Arai, K., S. Iwanaga, H. Toda, C. Genci, Y. Nishiyama, and M. Nakamura. 2011. Three-dimensional inkjet biofabrication based on designed images. *Biofabrication* 3 (3):034113. doi:10.1088/1758-5082/3/3/034113.
- Arslan-Yildiz, A., R. El Assal, P. Chen, S. Guven, F. Inci, and U. Demirci. 2016. Towards artificial tissue models: Past, present, and future of 3D bioprinting. *Biofabrication* 8 (1):014103. doi:10.1088/1758-5090/8/1/014103.
- Atala, A. 2009. Engineering organs. *Curr Opin Biotechnol* 20 (5):575–592. doi:10.1016/j.copbio.2009.10.003.
- Atala, A., S. B. Bauer, S. Soker, J. J. Yoo, and A. B. Retik. 2006. Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet* 367 (9518):1241–1246. doi:10.1016/S0140-6736(06)68438-9.
- Auger, F. A., L. Gibot, and D. Lacroix. 2013. The pivotal role of vascularization in tissue engineering. *Annu Rev Biomed Eng* 15:177–200. doi:10.1146/annurev-bioeng-071812-152428.
- Bottcher-Haberzeth, S., T. Biedermann, and E. Reichmann. 2010. Tissue engineering of skin. *Burns* 36 (4):450–460. doi:10.1016/j.burns.2009.08.016.
- Catros, S., J. C. Fricain, B. Guillotin, B. Pippenger, R. Bareille, M. Remy, E. Lebraud, B. Desbat, J. Amedee, and F. Guillemot. 2011. Laser-assisted bioprinting for creating on-demand patterns of human osteoprogenitor cells and nano-hydroxyapatite. *Biofabrication* 3 (2):025001. doi:10.1088/1758-5082/3/2/025001.
- Chung, J. H. Y., S. Naficy, Z. Yue, R. Kapsa, A. Quigley, S. E. Moulton, and G. G. Wallace. 2013. Bio-ink properties and printability for extrusion printing living cells. *Biomater. Sci.* 1.
- Dahl, S. L., A. P. Kypson, J. H. Lawson, J. L. Blum, J. T. Strader, Y. Li, R. J. Manson et al. 2011. Readily available tissue-engineered vascular grafts. *Sci Transl Med* 3 (68):68ra9. doi:10.1126/scitranslmed.3001426.
- de Leon, A., A. C. Barnes, P. Thomas, J. O'Donnell, C. A. Zorman, and R. C. Advincula. 2014. Transfer printing of self-folding polymer-metal bilayer particles. *ACS Appl Mater Interfaces* 6 (24):22695–22700. doi:10.1021/am5068172.
- Dvir, T., B. P. Timko, D. S. Kohane, and R. Langer. 2011. Nanotechnological strategies for engineering complex tissues. *Nat Nanotechnol* 6 (1):13–22. doi:10.1038/nnano.2010.246.
- Fagerholm, P., N. S. Lagali, K. Merrett, W. B. Jackson, R. Munger, Y. Liu, J. W. Polarek, M. Soderqvist, and M. Griffith. 2010. A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study. *Sci Transl Med* 2 (46):46ra61. doi:10.1126/scitranslmed.3001022.
- Ferris, C. J., K. G. Gilmore, G. G. Wallace, and M. In het Panhuis. 2013. Biofabrication: An overview of the approaches used for printing of living cells. *Appl Microbiol Biotechnol* 97 (10):4243–4258. doi:10.1007/s00253-013-4853-6.
- Gruene, M., A. Deiwick, L. Koch, S. Schlie, C. Unger, N. Hofmann, I. Bernemann, B. Glasmacher, and B. Chichkov. 2011a. Laser printing of stem cells for biofabrication of scaffold-free autologous grafts. *Tissue Eng Part C Methods* 17 (1):79–87. doi:10.1089/ten. TEC.2010.0359.

- Gruene, M., M. Pflaum, A. Deiwick, L. Koch, S. Schlie, C. Unger, M. Wilhelmi, A. Haverich, and B. N. Chichkov. 2011b. Adipogenic differentiation of laser-printed 3D tissue grafts consisting of human adipose-derived stem cells. *Biofabrication* 3 (1):015005. doi:10.1088/1758-5082/3/1/015005.
- Guillemot, F., A. Souquet, S. Catros, and B. Guillotin. 2010a. Laser-assisted cell printing: Principle, physical parameters versus cell fate and perspectives in tissue engineering. *Nanomedicine (Lond)* 5 (3):507–515. doi:10.2217/nnm.10.14.
- Guillemot, F., A. Souquet, S. Catros, B. Guillotin, J. Lopez, M. Faucon, B. Pippenger, R. Bareille, M. Remy, S. Bellance, P. Chabassier, J. C. Fricain, and J. Amedee. 2010b. High-throughput laser printing of cells and biomaterials for tissue engineering. *Acta Biomater* 6 (7):2494–500. doi:10.1016/j.actbio.2009.09.029.
- Guillotin, B., and F. Guillemot. 2011. Cell patterning technologies for organotypic tissue fabrication. *Trends Biotechnol* 29 (4):183–190. doi:10.1016/j.tibtech.2010.12.008.
- Highley, C. B., C. B. Rodell, and J. A. Burdick. 2015. Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels. *Adv Mater* 27 (34):5075–5079. doi:10.1002/adma.201501234.
- Hribar, K. C., P. Soman, J. Warner, P. Chung, and S. Chen. 2014. Light-assisted direct-write of 3D functional biomaterials. *Lab Chip* 14 (2):268–275. doi:10.1039/c3lc50634g.
- Hunsberger, J., O. Harrysson, R. Shirwaiker, B. Starly, R. Wusk, P. Cohen, J. Allickson, J. Yoo, and A. Atala. 2015. Manufacturing road map for tissue engineering and regenerative medicine technologies. *Stem Cells Transl Med* 4 (2):130–135. doi:10.5966/sctm.2014-0254.
- Kang, H. W., S. J. Lee, I. K. Ko, C. Kengla, J. J. Yoo, and A. Atala. 2016. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nat Biotechnol* 34 (3):312–319. doi:10.1038/nbt.3413.
- Keriquel, V., F. Guillemot, I. Arnault, B. Guillotin, S. Miraux, J. Amedee, J. C. Fricain, and S. Catros. 2010. In vivo bioprinting for computer- and robotic-assisted medical intervention: Preliminary study in mice. *Biofabrication* 2 (1):014101. doi:10.1088/1758-5082/2/1/014101.
- Kirchmayer, D. M. R. Gorkin III, M. in het Panhuis. 2015. An overview of the suitability of hydrogel-forming polymers for extrusion-based 3D-printing. *J Mater Chem B* 3:4105–4117.
- Koch, L., M. Gruene, C. Unger, and B. Chichkov. 2013. Laser assisted cell printing. *Curr Pharm Biotechnol* 14 (1):91–97.
- Koch, L., Kuhn, S., Sorg, H., Gruene, M., Schlie, S., Gaebel, R., Polchow, B. et al. 2010. Laser printing of skin cells and human stem cells. *Tissue Eng Part C Methods*. 16 (5):847–854.
- Kolesky, D. B., K. A. Homan, M. A. Skylar-Scott, and J. A. Lewis. 2016. Three-dimensional bioprinting of thick vascularized tissues. *Proc Natl Acad Sci USA* 113 (12):3179–3184. doi:10.1073/pnas.1521342113.
- Kolesky, D. B., R. L. Truby, A. S. Gladman, T. A. Busbee, K. A. Homan, and J. A. Lewis. 2014. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv Mater* 26 (19):3124–3130. doi:10.1002/adma.201305506.
- L'Heureux, N., T. N. McAllister, and L. M. de la Fuente. 2007. Tissue-engineered blood vessel for adult arterial revascularization. *N Engl J Med* 357 (14):1451–1453. doi:10.1056/NEJMc071536.
- Laschke, M. W., B. Vollmar, and M. D. Menger. 2009. Inosculation: Connecting the life-sustaining pipelines. *Tissue Eng Part B Rev* 15 (4):455–465. doi:10.1089/ten. TEB.2009.0252.
- Macchiarini, P., P. Jungebluth, T. Go, M. A. Asnaghi, L. E. Rees, T. A. Cogan, A. Dodson et al. 2008. Clinical transplantation of a tissue-engineered airway. *Lancet* 372 (9655):2023–2030. doi:10.1016/S0140-6736(08)61598-6.
- Marga, F., K. Jakab, C. Khatiwala, B. Shepherd, S. Dorfman, B. Hubbard, S. Colbert, and F. Gabor. 2012. Toward engineering functional organ modules by additive manufacturing. *Biofabrication* 4 (2):022001. doi:10.1088/1758-5082/4/2/022001.

- McCune, M., A. Shafiee, G. Forgacs, and I. Kosztin. 2014. Predictive modeling of post bioprinting structure formation. *Soft Matter* 10 (11):1790–1800.
- Mezel, C., A. Souquet, L. Hallo, and F. Guillemot. 2010. Bioprinting by laser-induced forward transfer for tissue engineering applications: Jet formation modeling. *Biofabrication* 2 (1):014103. doi:10.1088/1758-5082/2/1/014103.
- Miller, J. S., K. R. Stevens, M. T. Yang, B. M. Baker, D. H. Nguyen, D. M. Cohen, E. Toro, A. A. Chen, P. A. Galie, X. Yu, R. Chaturvedi, S. N. Bhatia, and C. S. Chen. 2012. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nat Mater* 11 (9):768–774. doi:10.1038/nmat3357.
- Mironov, V., R. P. Visconti, V. Kasyanov, G. Forgacs, C. J. Drake, and R. R. Markwald. 2009. Organ printing: Tissue spheroids as building blocks. *Biomaterials* 30 (12):2164–2174. doi:10.1016/j.biomaterials.2008.12.084.
- Mondy, W. L., D. Cameron, J. P. Timmermans, N. De Clerck, A. Sasov, C. Casteleyn, and L. A. Piegl. 2009. Computer-aided design of microvasculature systems for use in vascular scaffold production. *Biofabrication* 1 (3):035002. doi:10.1088/1758-5082/1/3/035002.
- Murphy, S. V., and A. Atala. 2013. Organ engineering—combining stem cells, biomaterials, and bioreactors to produce bioengineered organs for transplantation. *BioEssays* 35 (3):163–172. doi:10.1002/bies.201200062.
- Murphy, S. V., and A. Atala. 2014. 3D bioprinting of tissues and organs. *Nat Biotechnol* 32 (8):773–785. doi:10.1038/nbt.2958.
- Panwar, A., and L. P. Tan. 2016. Current status of bioinks for micro-extrusion-based 3D bioprinting. *Molecules* 21 (6):685. doi:10.3390/molecules21060685.
- Paulsen, S. J., and J. S. Miller. 2015. Tissue vascularization through 3D printing: Will technology bring us flow? *Dev Dyn* 244 (5):629–640. doi:10.1002/dvdy.24254.
- Pepper, M. E., V. Seshadri, T. Burg, B. W. Booth, K. J. Burg, and R. E. Groff. 2011. Cell settling effects on a thermal inkjet bioprinter. *Conf Proc IEEE Eng Med Biol Soc* 2011:3609–3612. doi:10.1109/IEMBS.2011.6090605.
- Pepper, M. E., V. Seshadri, T. C. Burg, K. J. Burg, and R. E. Groff. 2012. Characterizing the effects of cell settling on bioprinter output. *Biofabrication* 4 (1):011001. doi:10.1088/1758-5082/4/1/011001.
- Raya-Rivera, A. M., D. Esquiliano, R. Fierro-Pastrana, E. Lopez-Bayghen, P. Valencia, R. Ordorica-Flores, S. Soker, J. J. Yoo, and A. Atala. 2014. Tissue-engineered autologous vaginal organs in patients: A pilot cohort study. *Lancet* 384 (9940):329–336. doi:10.1016/S0140-6736(14)60542-0.
- Raya-Rivera, A., D. R. Esquiliano, J. J. Yoo, E. Lopez-Bayghen, S. Soker, and A. Atala. 2011. Tissue-engineered autologous urethras for patients who need reconstruction: An observational study. *Lancet* 377 (9772):1175–1182. doi:10.1016/S0140-6736(10)62354-9.
- Shin'oka, T., Y. Imai, and Y. Ikada. 2001. Transplantation of a tissue-engineered pulmonary artery. *N Engl J Med* 344 (7):532–533. doi:10.1056/NEJM200102153440717.
- Tirella, A., F. Vozzi, C. De Maria, G. Vozzi, T. Sandri, D. Sassano, L. Cognolato, and A. Ahluwalia. 2011. Substrate stiffness influences high resolution printing of living cells with an ink-jet system. *J Biosci Bioeng* 112 (1):79–85. doi:10.1016/j.jbiosc.2011.03.019.
- Varkey, M., and Atala, A. 2015. Organ bioprinting: A closer look at ethics and policies. *WF J Law and Policy* 5 (2):275–298.
- Visscher, D. O., E. Farre-Guasch, M. N. Helder, S. Gibbs, T. Forouzanfar, P. P. van Zuijlen, and J. Wolff. 2016. Advances in bioprinting technologies for craniofacial reconstruction. *Trends Biotechnol* 34 (9):700–710. doi:10.1016/j.tibtech.2016.04.001.
- Yu, Y., Y. Zhang, J. A. Martin, and I. T. Ozbolat. 2013. Evaluation of cell viability and functionality in vessel-like bioprintable cell-laden tubular channels. *J Biomech Eng* 135 (9):91011. doi:10.1115/1.4024575.



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